Universidade de Aveiro Departamento de Biologia
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Biodegradação Anaeróbia do Ácido Oleico

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dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Métodos Biomoleculares Avançados, realizada sob a orientação científica da Professora Doutora Madalena Alves, Professora Associada do Departamento de Engenharia Biológica da Universidade do Minho e da Professora Doutora Etelvina Figueira, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave

Biodegradação; ácido oleico; AGCL;DGGE; consórcios anaeróbios; ecologia molecular.

resumo

O ácido oleico (C18:1) é um Ácido Gordo de Cadeia Longa (AGCL), considerado especialmente problemático em processos de digestão anaeróbia uma vez que exerce um efeito inibitório sobre os microrganismos e adsorve aos agregados microbianos causando a sua flutuação e consequente "washout" em reactores anaeróbios de alta carga. Nesta tese, após uma introdução geral onde é apresentado o estado da arte, descrevem-se três estudos sobre biodegradação anaeróbia de ácido oleico. No capítulo 2, foi estudada uma biomassa altamente carregada (5985 mg CQO.g SV^{-1}). Avaliou-se a sua capacidade de degradar oleato, em "batch", em concentrações entre 100 e 1500 mg/l. As fases "lag" que precederam o início da produção de metano aumentaram com o aumento da concentração, até um periodo de 17 dias verificado para a concentração mais elevada. A recuperação da CQO-metano não excedeu os 50% nas concentrações mais elevadas de 1000 e 1500 mg/l. Após a mineralização do substrato associado à biomassa, e para as mesmas concentrações, obtiveram-se fases "lag" máximas de 3 dias e recuperações da CQO-metano na ordem dos 80%, evidenciando o aumento da capacidade da biomassa em degradar oleato. A actividade metanogénica específica em acetato e em H_2/CO_2 como substratos individuais aumentou 2,6 e 2 vezes respectivamente, após a mineralização dos substrato associado à biomassa, confirmando a reversibilidade do efeito inibitório dos AGCL. No capítulo 3 efectuou-se o estudo dos intermediários da degradação do ácido oleico em ensaios "batch". As duas biomassas utilizadas foram recolhidas do mesmo digestor mas uma delas foi aclimatizada aos AGCL por contacto num reactor à escala laboratorial durante 50 dias,enquanto que a outra biomassa não contactou previamente com AGCL. Os intermediários da degradação foram analisados nas fases líquida e sólida durante 30 dias. De um modo geral, e para as duas biomassas, verificou-se adsorção do ácido oleico à fase sólida durante os primeiros 50 minutos, sendo depois convertido a metano. No caso da biomassa não aclimatizada, a taxa de degradação foi aproximadamente linear não se verificando acumulação apreciável de acetato durante o ensaio. No caso da biomassa aclimatizada, após um periodo de 10 dias foi observado um decréscimo acentuado da quantidade do oleato seguido de uma acumulação transiente de acetato que foi posteriormente convertido a metano. Foram verificadas fases "lag" antes de se iniciar a produção de metano nos ensaios com as biomassas aclimatizada e não aclimatizada, mas após o início da produção de metano, o tempo necessário para mineralizar 830 mg/l de oleato foi de 5 e 12 dias, respectivamente. No capítulo 4, as alterações da comunidade microbiana desenvolvida num reactor durante o tratamento de um efluente contendo oleato, através da análise do gene 16S rRNA pelas técnicas de PCR-DGGE, revelou que a composição da comunidade bacteriana foi mais afectada do que a comunidade de arquaea.

keywords

Biodegradation; oleic acid; LCFA; DGGE; anaerobic consorcia; molecular ecology.

abstract

Oleic Acid (C18:1) is an unsaturated Long Chain Fatty Acid (LCFA), described as especially problematic in anaerobic digestion processes due to its microbial inhibitory effect as well as because it adsorbs onto microbial aggregates causing flotation and washout in high-rate anaerobic reactors. In this thesis, after a general introduction referring the context and motivation and presenting the state of the art, three experiments are described about anaerobic biodegradation of oleic acid. In chapter 2, a sludge heavily loaded with 5985 mg COD.g VS^{-1} was studied in terms of its capacity to biodegrade oleic acid in batch assays, in concentrations ranging from 100 to 1500 mg/l. Lag phases before the onset of methane production increased with the oleic acid concentration, up to 17 days for 1500 mg/l and methane recovery did not exceed 50% for the highest concentrations tested (1000 and 1500 mg/l). After the mineralization of the biomass-associated substrate, and for the same concentration range, maximum lag phases of three days and methane recoveries up to 80% were obtained, evidencing an improving capacity of the sludge to biodegrade oleic acid. Also the specific methanogenic activity with acetate and H_2/CO_2 as individual substrates increased 2.6 and 2 times respectively, after the mineralization of the biomass associated substrate, confirming the reversibility of the inhibitory effect of LCFA. In chapter 3, the intermediates of oleic acid degradation were also studied in batch assays. Two different sludges were sampled from the same digester but afterwards one was acclimated to LCFA by contact in a lab-scale reactor during around 50 days and the other one was not acclimated. LCFA intermediates were analyzed in the liquid and in the solid phases along a 30 days experiment. In general, for both sludges, oleic acid adsorbed to the solid phase in the first 50 minutes and was converted to methane afterwards. In the non-acclimated sludge the degradation rate was approximately linear and there was not an appreciable accumulation of acetate during the course of the experiment. In the acclimated sludge, after an initial period of 10 days a sharp decrease on oleic acid quantity in the solid phase was observed followed by a significant, but transient accumulation of acetate that was afterwards converted to methane. Although both sludges exhibited a lag phase before the onset of methane production, the acclimated one was able to mineralize 830 mg/l of oleic acid in 5 days whereas the non-acclimated one took around 12 days.

The study of the anaerobic microbial community dynamics during discontinuous operation of a lab-scale reactor treating a synthetic dairy wastewater was also investigated by analysis of the 16S rRNA gene using the polymerase chain reaction (PCR)-based denaturing gradient gel electrophoresis (DGGE) method. The composition of the bacterial community, based on the analysis of the DGGE patterns, was more affected than the archaeal population.

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AD	Anaerobic digestion
ATP	Adenosine triphosphate
bp	Base pair
COD	Chemical oxygen demand
$\Delta G^{0'}$	Standard free energy change
DGGE	Denaturing gradient gel electrophoresis
DOC	Dissolved organic carbon
DNA	Deoxyribonucleic acid
EGSB	Expanded granular sludge bed
FAD	Flavinadenine dinucleotide
GC	Guanine/ cytosine
GC	Gas chromatography
HPLC	High performance liquid chromatography
HRT	Hydraulic retention time
HS-HTP	Mercaptoheptanoylthreonine phosphate
IC	Internal circulation
LCFA	Long chain fatty acid
MR	Methyl-coenzyme-M reductase
MFR	Methanofuran
mRNA	Messenger ribonucleic acid

NAD	Nicotinamide-adenine dinucleotide
OHPA	Obligate hydrogen-producing acetogens
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SMA	Specific methanogenic activity
SSU	Small subunit
STP	Standard temperature and pressure conditions
TS	Total solids
UASB	Upflow anaerobic sludge blanket
VFA	Volatile fatty acid
VS	Volatile solids
w/v	Weight/volume

1 General Introduction

1.1 Context and Motivation

Implementation of Anaerobic Digestion (AD) technology was enhanced at a large pace in the last years due to the development of new class of high rate upflow reactors based on the formation of anaerobic granular sludge. The huge success of the Upflow Anaerobic Sludge Blanket (UASB) reactors as well as the subsequent technologies such as the Expanded Granular Sludge Bed (EGSB) and the Internal Circulation (IC) reactors is in part due to the increasing knowledge on the biochemical and microbiological basis of the anaerobic process. These technologies are however limited to a class of simple, soluble and acidified or partially acidified substrates, being 87% of the industrial market concentrated on breweries and beverages, pulp and paper, distilleries and food industry wastewater treatment. High rate anaerobic reactors for complex wastewater containing appreciable amounts of lipids do not exist in the market. Probably the reason is because lipids were considered for years as difficult compounds that originated long chain fatty acids, which were described in the literature as toxic and even bactericidal (Angelidaki and Ahring, 1992, Rinzema *et al*., 1994). In the research group on Environmental Biotechnology at the Department of Biological Engineering, University of Minho, research on anaerobic degradation of Long Chain Fatty Acids started some years ago, and provided clear evidences that LCFA can be efficiently biodegraded under anaerobic conditions. The obtained results proved that: (i) LCFA do not exert a permanent toxic effect on anaerobic consortia (Pereira *et al*., 2002, 2003); (ii) LCFA can be efficiently mineralized to methane in some defined conditions (Pereira *et al*., 2004); (iii) Accumulation of LCFA on the microbial aggregates by mechanisms of adsorption, precipitation and entrapment causes a reversible physical inhibition that limits the access of substrates to the cells (Pereira *et al*., 2005); (iv) The anaerobic consortia that degrades saturated and unsaturated LCFA show a different microbial composition (Sousa *et al*., 2007a). *Syntrophomonas zehnderi* sp., a new oleic acid degrading synthrophic bacteria was isolated (Sousa *et al*., 2007b) in co-culture with *Methanobacterium formicicum*. A patent of a new high rate anaerobic reactor for the treatment of complex effluents with lipids was registered and the proof of concept is in progress (PCT/PT2005/000020 – Alves *et al*., 2005).

The present work provides a contribution for a better understanding of oleic acid anaerobic degradation. After a general introduction and state of the art, presented in this chapter, the thesis describes and discusses the results obtained in three experiments, presented in Chapters 2 to 4:

Chapter 2 - Degradation of oleic acid in batch assays by a sludge heavily loaded with LCFA;

Chapter 3 - Fate of intermediates of oleic acid degradation in the liquid and solid phases, during the batch degradation of oleic acid;

Chapter 4 - Microbial community dynamics during the discontinuous operation of a reactor treating a synthetic dairy wastewater.

1.2 Overview of Anaerobic Digestion

1.2.1 Biochemical and microbial fundamentals

Anaerobic treatment processes are complex ecosystems comprising several diverse microbial guilds that work together, in a coordinated manner, to convert the organic components to methane and carbon dioxide.

Key organisms in the conversion of complex organic materials to methane are fermenters, especially the H_2-producing fatty acid-oxidizing bacteria, which use fatty acids or alcohols as energy sources. These syntrophic organisms grow poorly or not at all on these substrates in pure culture but, in association with H_2-consuming organisms, they are capable to grow luxuriantly. Hydrogen consumption by methanogens is critically important to the growth of H_2-producing fatty acid-oxidizing bacteria. The genus *Syntrophomonas* and *Syntrophobacter* include organisms dependent on syntrophic relationships for growth (Madigan *et al.*, 1997).

The first step of anaerobic digestion consists in the hydrolysis of biopolymers. Bacteria from hydrolytic genera such as *Clostridium*, *Peptococcus*, *Vibrio*, *Micrococcus* and *Bacillus* produce a number of extracellular enzymes that are capable of initiating the attack on the complex substrates (lipids, proteins and carbohydrates) into simple

components (fatty acids, glycerol, peptides, amino acids, oligosaccharides and sugars). These enzymes include protease, lipase, cellulase, pectinase, amylase and chitinase. The relative composition and activity of these enzymes reflect the prevalence of their respective substrates in the digester feed. The extracellular enzymes are able to access large substrate molecules that are incapable of crossing the bacterial cell wall due to their size (Madigan *et al.*, 1997).

Three main mechanisms have been suggested for the release of enzymes and the subsequent hydrolysis of complex substrates during anaerobic digestion. In the first mechanism, the organism secretes enzymes to the bulk liquid, where they will either adsorb to a particle or react with a soluble substrate. In the second mechanism, the organism attaches to the particle, secretes enzymes into the vicinity of the particle and benefits from the released dissolved substrates. In the third mechanism, the organism has an attached enzyme, in the outer cell membrane, that may also act as a transport receptor to the interior of the cell. Therefore, a good contact between biomass and substrate seems to be a prerequisite to the hydrolysis. The accumulation of LCFA at the lipid-water interface causes inhibition of the lipase activity by physical-chemical changes of the interface such as surface tension changes. The hydrolysis rate depends on the physical state and structure of the substrate and its accessibility for hydrolytic enzymes and also on the rate of microbial attachment to the substrate, which depends on the type of microorganisms (Angelidaki & Sanders, 2004).

The monomers produced by the hydrolytic bacteria are fermented during the second acid-forming stage to produce several intermediary products, namely acetate, propionate, butyrate and hydrogen. There are two groups of acid-forming bacteria, the acidogenic and the acetogenic bacteria. The acidogenic bacteria provide important substrates for acetogens and methanogens namely acetate, hydrogen and carbon dioxide. This stage includes many different fermentative genera such as *Clostridium*, *Syntrophomonas*, *Lactobacillus*, *Streptococcus*, *Pseudomonas*, *Micrococcus*, *Bacteroids*, *Bacillus* and *Escherichia* among others. The facultative members of this group help to protect the oxygen sensitive methanogens by consuming traces of oxygen that may enter in the feed.

In the acetogenesis step, bacteria produce acetate, carbon dioxide and hydrogen. Two distinct groups of acetogenic bacteria can be distinguished on the bases of their

metabolism, the obligate hydrogen-producing acetogens (OHPA) and the homoacetogens. OHPA, also called proton-reducing acetogens, produce acetic acid, carbon dioxide and hydrogen from the major fatty acid intermediates (propionate and butyrate), alcohols and other higher fatty acids (valerate, isovalerate, stearate, palmitate and myristate) via β-oxidation. These bacteria are particularly important in the β-oxidation of long chain fatty acids, arising from lipid hydrolysis and are also involved in the anaerobic degradation of aromatic compounds. *Syntrophomonas wolfei* and *Syntrophomonas wolinii* are OHPA species which oxidize butyrate and propionate, respectively.

Homoacetogens catalyze the formation of acetate from hydrogen and carbon dioxide. Homoacetogens are known in the genera *Acetobacterium*, *Acetoanaerobium*, *Acetogenium*, *Butribacterium*, *Clostridium* and *Pelobacter*. Homoacetogenic bacteria are also syntrophs because they participate in the interspecies hydrogen transfer process which maintains the low hydrogen concentrations required by the OHPA (Anderson *et al*., 2003). Since the growth rates of syntrophic fatty acid oxidizers are generally very slow, as soon as H_2 is formed during the fermentations, it is quickly consumed by a methanogen, a homoacetogen or a sulphate reducer. *Acetobacterium woodii* and *Clostridium aceticum* are examples or homoacetogenic organisms (Madigan *et al.*, 1997).

Ultimately, methanogens convert acetate, H_2 and CO_2 to methane (Figure 1.1).

In the late 1970s, Woese *et al.* proposed the existence of three domains (Bacteria, Archaea and Eucarya), based on the comparison of the oligonucleotides generated by RNase digestions of 16S rRNAs. Each domain comprises two or more kingdoms. Archaea is formally subdivided into the kingdoms Euryarchaeota (encompassing the methanogens and their phenotypically diverse relatives) and Crenarchaeota (including the extremely thermophilic archaebacteria). Methanogens are strict anaerobes and form methane as the end-product of their metabolism. Methane production is considered to be the slowest (rate limiting) step in the anaerobic digestion process. Moreover, since the methanogens are most active in the pH range of 6.5-8.0, they will be sensitive in environments poorly buffered against acidification caused by the products of the acidogenic and acetogenic bacteria (Anderson *et al*., 2003). Some substrates that can be utilized by methanogens and the overall reactions of methane formation are listed in Table 1.1.

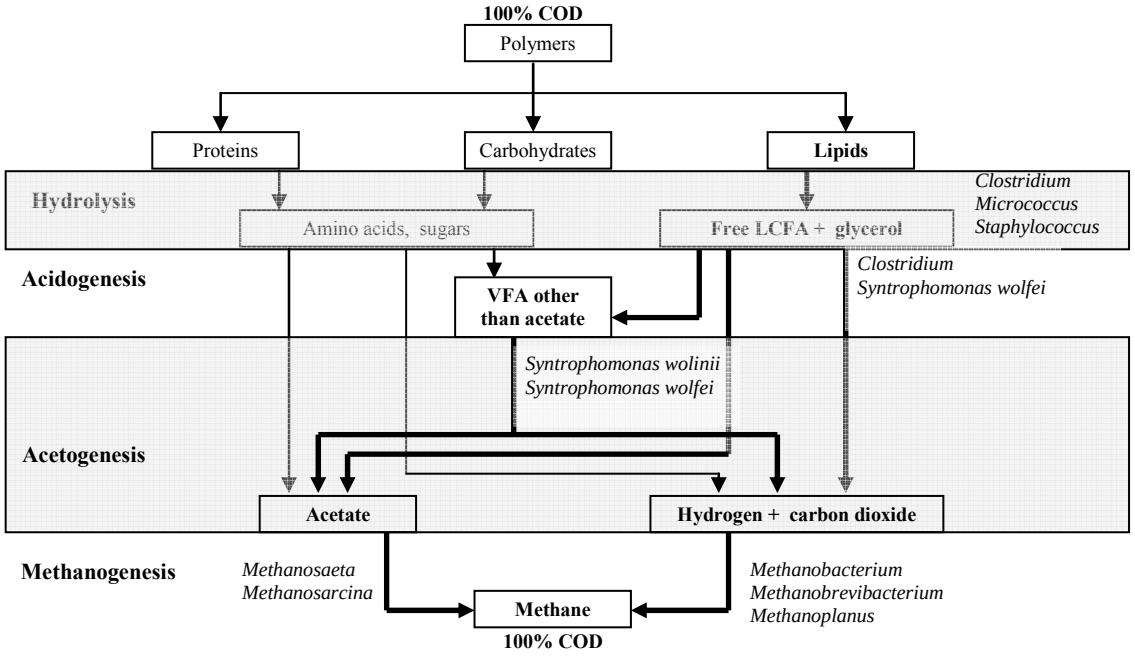


Figure 1.1 Carbon flow to methane in anaerobic digesters with special emphasis (dark arrows) in lipid degradation pathway and with the indication of some organisms responsible for each step. Adapted from Salminen *et al.*, 2002 and Anderson *et al*., 2003.

Methanogenesis is a low-redox process that yields small amounts of energy during the reduction of specific substrates (Table 1.1) to methane (CH_4). Methane synthesis *per se* is catalyzed by methyl-coenzyme-M reductase (MR). The CH_3 group of methyl-coenzyme-M (CH_3-S-CoM) is reduced to CH_4 by reductant transferred from a methanogen specific cofactor, mercaptoheptanoylthreonine phosphate (HS-HTP). During the dissimilation of methanol, methylamines, or acetate to methane, methyl groups are transferred intact from these substrates to coenzyme-M (HS-CoM). On the other hand, methanogenesis from CO, CO_2, or formate requires first a series of reductive steps to generate the CH_3 group. CO_2 is fixed initially to the cofactor methanofuran (MFR) to produce formyl-MFR. The formyl group is then transferred to a second cofactor, tetrahydromethanopterin (H_4MPT), which carries the C1 moiety during its sequential reduction through the methenyl and methylene levels to the methyl level. The methyl group is then transferred to HS-CoM to form CH_3-S-CoM, the substrate for MR. Methyl group reduction to CH_4 by MR also results in the synthesis of a heterodisulfide (CoM-S-S-HTP), which must be reduced to regenerate the

cofactors HS-CoM and HS-HTP, needed for continued methanogenesis (Palmer & Reeve, 1993).

Table 1.1 Substrates converted to methane by various methanogenic Archaea (adapted from Madigan *et al.*, 1997).

Substrate type	**Name**	**Reaction**	**$\Delta G^{0'}$ (kJ/reaction)**
CO_2-type substrates	Carbon dioxide[a]	$CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$	- 131
	Formate	$4\ HCOO^- + 4\ H^+ \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$	- 145
	Carbon monoxide	$4\ CO + 2\ H_2O \rightarrow CH_4 + 3\ CO_2$	- 210
Methyl substrates	Methanol	$4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$	- 319
	Methylamine	$4\ CH_3NH_3Cl + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_4Cl$	- 230
Acetotrophic substrate	Acetate	$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	-31

[a] With electrons derived from H_2.

According to their substrate specificity, methanogens are divided into two groups, the acetoclastic methanogens and the hydrogen-utilizing methanogens.

Only two methanogenic genera contain species that are able to utilize acetate and these are *Methanosaeta* (or *Methanothrix*) and *Methanosarcina*. In addition to the acetoclastic activity, *Methanosarcina* spp. are also capable of using methanol, methylamines and sometimes H_2/CO_2 as growth substrates, while *Methanosaeta* spp. are restricted to growth only on acetate. Acetate is regarded as the source of up to 70% of methane evolved in digesters (Hwu & Lettinga., 1997; Anderson *et al*., 2003). On the other hand, hydrogen-utilizing methanogenic bacteria are responsible for up to 30% of the total methane production within anaerobic digesters. These methanogens reduce carbon dioxide, formate, methanol and methylamines, using the hydrogen produced fermentatively by the hydrolytic and acid-forming bacteria earlier in the digestion process. *Methanobacterium*, *Methanobrevibacter*, *Methanothermus*, *Methanococcus*, *Methanomicrobium*, *Methanogenium* and *Methanospirillum* are examples of genera of hydrogen-utilizing

methanogenic bacteria (Anderson *et al*., 2003). More recently, several species have been characterized using molecular tools such as *Methanocaldococcus jannaschii*, *Methanothermobacter thermautotrophicus*, *Methanopyrus kandleri*, *Methanococcus maripaludis*, *Methanobrevibacter acididurans* (Templer *et al.*, 2006).

1.2.2 Fats, oils and fatty acids

Fats and oils exist mainly in the form of triacylglycerols, in which fatty acid molecules are linked by ester bonds to the three hydroxyl groups of glycerol (Madigan *et al.*, 1997; Taiz & Zeiger, 1998; Templer *et al.*, 2006). Usually the term fat applies to solid and oils are liquid at room temperature. In the form of triacylglycerols, fatty acids are found in both plants and animals, where they are biosynthesized from acetate through the pathway of acetyl coenzyme A. The three acyl groups in a triacylglycerol may be similar or different.

Fatty acids are classified as saturated, monounsaturated or polyunsaturated (Templer *et al.*, 2006). Most naturally occurring fatty acids possess an even number of carbon atoms and an unbranched carbon chain (Table 1.2). The carbon chain can be completely saturated or may incorporate one or more multiple bonds. When double bonds are present, they almost always have the cis (or Z) configuration. Acyl groups containing 14 to 20 carbon atoms are the most abundant in triacylglycerols (Carey, 1996).

Sodium oleate has a polar carboxylate group at one end of the long hydrocarbon chain. The carboxylate group is hydrophilic and confers water solubility on the molecule. The hydrocarbon chain is lipophilic and tends to associate to other hydrocarbon chains. When sodium oleate is placed in water forms a colloidal dispersion of spherical aggregates called micelles. Each micelle is composed of 50 to 100 individual molecules. Micelles are formed spontaneously when the carboxylate concentration exceeds a certain minimum value, called the critical micelle concentration. Because micelles surfaces are negatively charged, they repel each others rather than clustering to form higher aggregates (Carey, 1996).

Table 1.2 Some representative fatty acids.

Common name (C atoms: double bonds)	**Systematic name**	**Structure**
Lauric acid (12:0)	Dodecanoic acid	$CH_3(CH_2)_{10}CO_2H$
Myristic acid (14:0)	Tetradecanoic acid	$CH_3(CH_2)_{12}CO_2H$
Palmitic acid (16:0)	Hexadecanoic acid	$CH_3(CH_2)_{14}CO_2H$
Stearic acid (18:0)	Octadecanoic acid	$CH_3(CH_2)_{16}CO_2H$
Arachidic acid (20:0)	Icosanoic acid	$CH_3(CH_2)_{18}CO_2H$
Oleic acid (18:1)	(Z)-9-Octadecenoic acid	$CH_3(CH_2)_7CH{=}CH(CH_2)_7CO_2H$
Linoleic acid (18:2)	(9Z, 12Z)-9,12-Octadecadienoic acid	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7CO_2H$
Linolenic acid (18:3)	(9Z, 12Z, 15Z)-9,12,15-Octadecatrienoic acid	$CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_7CO_2H$
Arachidonic acid (20:4)	(5Z, 8Z, 11Z, 14Z)-5,8,11,14-Icosatetraenoic acid	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_3CO_2H$

LCFA are present as salts (soaps) in pH neutral ranges. Thus, they exert a surface-active effect and in aqueous systems behave like synthetic surfactants. The unionised form of LCFAs adsorbs initially to the microbial cell surface (Hwu & Lettinga, 1997; Salminen *et al.*, 2002). At neutral pH, e.g. in a bioreactor, LCFA become ionised, being appropriate to refer to them in that form, for instance oleate instead of oleic acid. Both terms are used throughout the present thesis.

Because lipid hydrolysis is a fast process under methanogenic conditions, it is reasonable to expect that LCFA, which contain approximately 95% of the original lipid COD, will prevail in lipid-containing wastewaters (Hanaki *et al.*, 1981). Palmitic acid

(hexadecanoic) and oleic acid (cis-9-octadecenoic) are, respectively, the most abundant saturated and unsaturated LCFA present in waste/wastewater (Table 1.3).

Table 1.3 LCFA composition in various common raw materials and wastewater (% of total LCFA). (Pereira, 2003c)

Common name (structure[1])	Palm oil [a]	Olive oil [a]	Soya bean oil [a]	Cotton seed oil [a]	Cocoa butter [a]	Whole milk [b]	Chicken fat [a]	Beef tallow [a]	Raw sewage [c]	Domestic wastewater [d]
Lauric (12:0)						7.0		1.0		
Myristic (14:0)	1.4		1.0	1.4		6.0	1.4	2.6		2.2
Palmitic (16:0)	42.9	14.3	11.0	25.7	26.7	21.0	21.0	28.1	27.6	16.4
Palmitoleic (16:1)	0.7	1.4		1.0	0.5	2.0	6.7	3.8		0.9
Stearic (18:0)	4.8	2.4	4.8	2.9	32.9	6.0	4.3	20.0	16.7	8.1
Oleic (18:1)	39.0	71.4	21.9	15.2	33.8	39.0	42.4	37.6	48.3	30.5
Linoleic (18:2)	10.0	5.5	49.0	51.9	4.3	13.0	20.0	2.9	5.1	29.2

[1]Number of carbon atoms:number of double bonds.

[a]Taylor, 1965; [b]Hanaki *et al*., 1981; [c]Viswanathan *et al*., 1962; [d]Quéméneur and Marty, 1994.

1.2.3 Bacterial long chain fatty acid transport and ß-oxidation

Several studies concerning genetic and biochemical aspects of LCFA transport have been done in the last years, using *E. coli* as a model organism.

The transport of LCFAs represents a fundamental biological process given the energetic cost of fatty acid synthesis and the necessity of obtaining essential fatty acids from the environment. The biochemical mechanisms behind fatty acid transport involve specific proteins and enzymes, which act either directly at the membrane or indirectly at the level of downstream metabolism. The transport and activation of exogenous LCFAs lead to changes in transcription patterns of the genes encoding the proteins required for fatty acid biosynthesis and degradation (DiRusso & Black, 2004). DiRusso and Black reported that under normal conditions the biosynthesis of LCFA occurs. On the other hand, when the cells encounter LCFAs in the environment, the biosynthesis is repressed and the

uptake of exogenous LCFA and its subsequent degradation takes place. Figure 1.2 shows a schematic representation of LCFA uptake by an *E. coli* cell, as well as the intracellular LCFA degradation via β-oxidation and Figure 1.3 describes in detail the classical pathway of β-oxidation of oleoyl-CoA also in *E. coli*. The proteins involved are also represented.

β-oxidation of long chain fatty acids

Microorganisms utilize long-chain carboxylic acids only after hydrolysis of the ester bond by extracellular lipases. The resulting glycerol may enter the glycolytic pathway after oxidation and phosphorylation as glyceraldehyde-3-phosphate and fatty acids are further degraded via β-oxidation pathway (Madigan *et al.*, 1997; Becker & Märkl, 2000). In β-oxidation process, two carbon atoms of the fatty acid are split off at a time. The prokaryotic enzymes involved are cytoplasmic. The fatty acid is first activated with coenzyme A. Oxidation results in the release of acetyl-CoA and the formation of a fatty acid shorter by two carbons. This process repeats and another acetyl-CoA molecule is released. Two dehydrogenation reactions occur. In the first, electrons are transferred to flavinadenine dinucleotide (FAD) and in the second, they are transferred to NAD^+. Fatty acids are good electron donors. The anaerobic oxidation of palmitic acid results in the net synthesis of 129 ATP molecules from electron transport phosphorylation, from electrons generated during the formation of acetyl-CoA from beta oxidations and from oxidation of acetyl-CoA units themselves through the citric acid cycle (Madigan *et al.*, 1997; Salminen *et al.*, 2002).

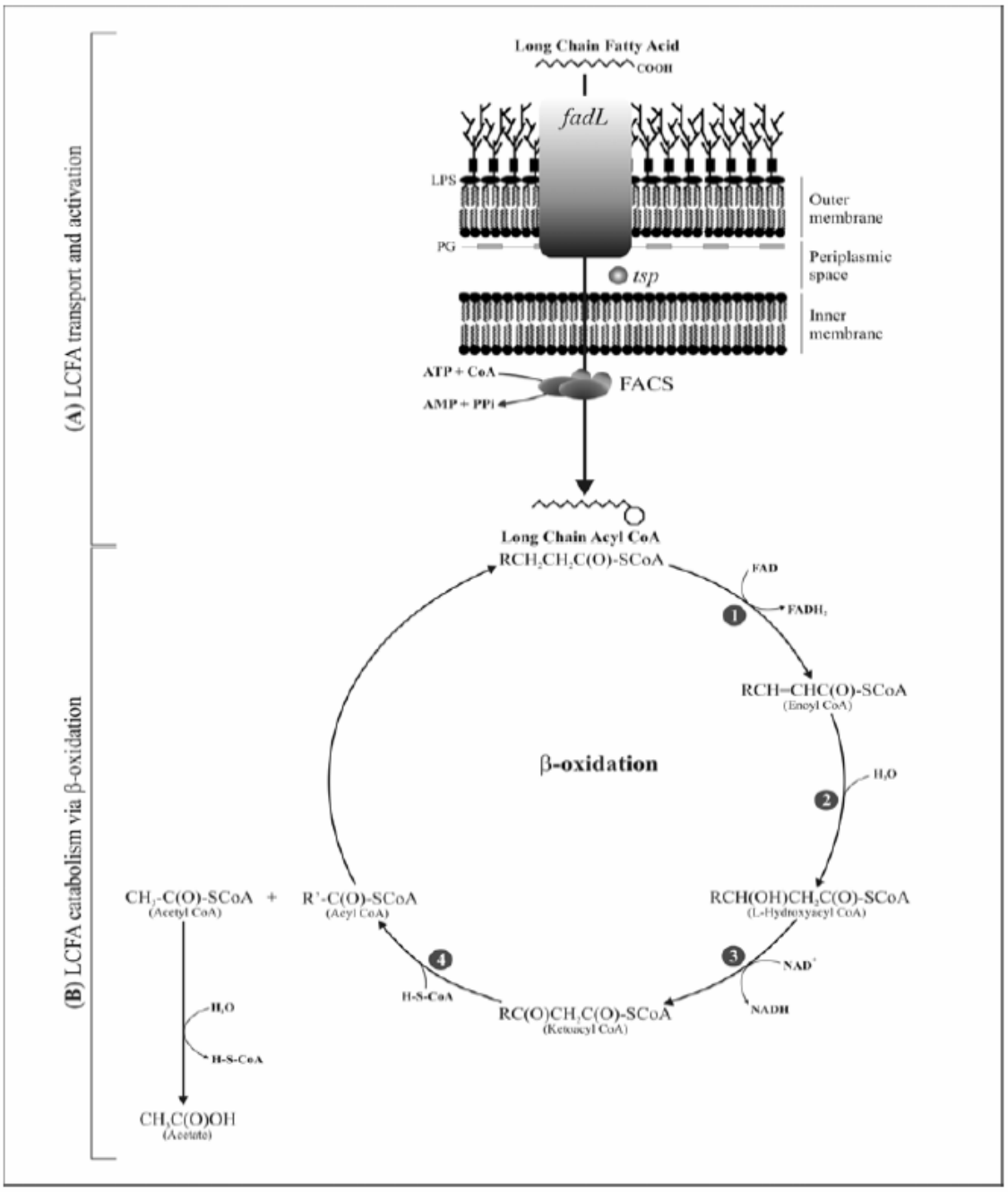


Figure 1.2 LCFA transport and catabolism in *Escherichia coli*. **(A)** Coupled transport and activation of exogenous LCFA. Central components of this system include FadL, an outer membrane-bound fatty acid transport protein, FadD, an inner membrane-associated long chain acyl-CoA synthetase, and FACS, a long chain acyl-CoA responsive transcription factor. **(B)** LCFA degradation via cyclic β-oxidation. Enzymes catalyzing each of the numbered reactions of the pathway (and respective encoding genes) are: **1**- acyl CoA dehydrogenase (*fadFG*) and electron transferring flavoprotein (*fadE*); **2**- enoyl CoA hydratase (*fadB*); **3**- L-3 hydroxyacyl dehydrogenase (*fadB*); and, **4**- β-ketothilose (*fadA*). (Sousa, 2007c)

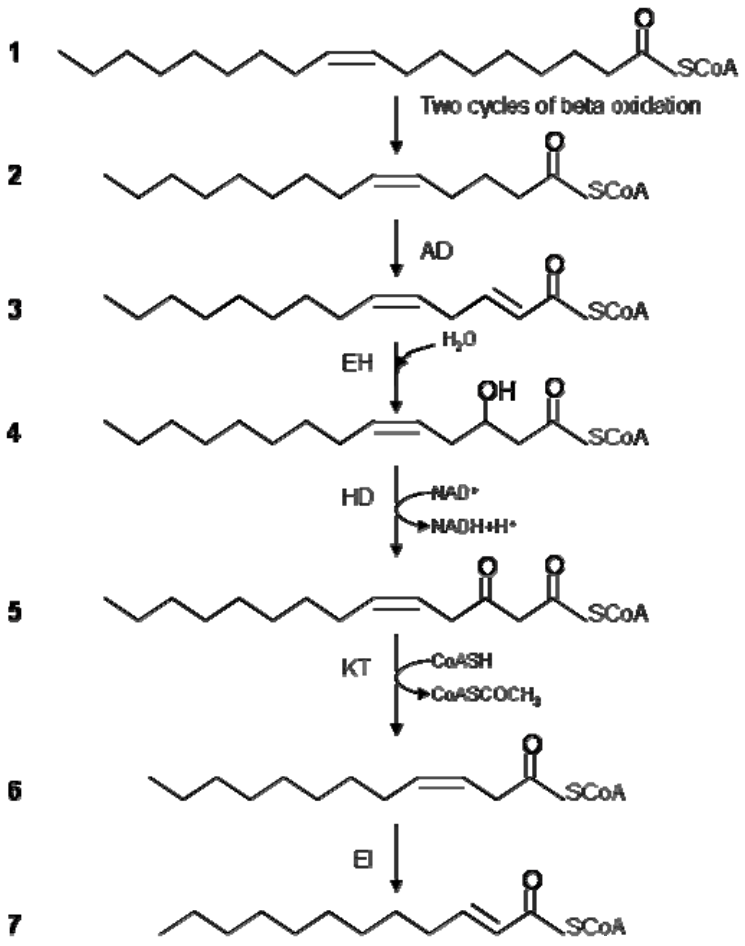


Figure 1.3 Representation of the classical pathway of β-oxidation of oleoyl-CoA in *E. coli*. AD, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HD, L-3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase; EI, Δ^3, Δ^2 - enoyl-CoA isomerase. (Adapted from Ren *et al.*, 2004)

Under anaerobic conditions the pathway of oleic acid β-oxidation is not completely clear. In 1976, Weng and Jeris proposed a mechanism where the double bond is first hydrogenated originating stearic acid and then the β-oxidation proceeds towards acetate. However, stearic acid was never detected as an intermediate of oleate degradation. In *E. coli*, a first hydrogenation was not suggested (Ren *et al.*, 2004).

1.3 Anaerobic Treatment of LCFA based Wastewaters

The overall objective of biological treatment of industrial wastewaters is to remove or reduce the concentration of organic and inorganic compounds. Because some of the constituents and compounds found in industrial wastewater are toxic to microorganisms, pre-treatment may be required before the wastewater can be discharged to a municipal collection system (Metcalf & Eddy, 2003).

The dairy industry produces many different products such as pasteurized, condensed, skimmed and powered milk, yogurts, butter and cheese. The main contributors to the organic load of these wastewaters are lactose, fats and proteins (Vidal *et al*., 2000).

Many problems are encountered during the anaerobic treatment of lipid-containing wastewaters such as inhibition and sludge flotation and washout, in part due to the adsorption of LCFA to the microbial cell walls. The inhibitory effect of LCFA towards microorganisms depends on a variety of factors specifically the LCFA concentration, the temperature (methanogenesis under thermophilic conditions being more susceptible to oleate toxicity than under mesophilic conditions) (Hwu & Lettinga, 1997), the carbon chain length and the degree of LCFA saturation. Saturated LCFAs with 12-14 carbon atoms and unsaturated LCFAs with 18 carbon atoms are considered the most inhibitory (Salminen & Rintala, 2002). Also, some groups of microorganisms are more sensitive than others, for example, Gram-positive bacteria and methanogens are more vulnerable to LCFAs than Gram-negative bacteria (Koster & Cramer, 1987) and acetate-utilizers seem to be more affected by LCFA than H_2-utilizers (Hanaki *et al.*, 1981). Furthermore, the inhibition depends on the specific surface area of sludge with suspended sludge being more sensitive than granular sludge (Alves *et al.*, 2001b; Salminen & Rintala, 2002). Although more resistant, granulation and/or physical stability of granules is critical when treating lipid containing wastewaters (Alves *et al*., 2001a; Pereira *et al.*, 2002).

The inhibition of substrates degradation, other than LCFA, by oleate has been reported by several authors. Those substrates include glucose, its degradation being inhibited by oleate concentrations equal or greater than 300 mg/l (Alosta *et al*., 2004), hydrogen, by oleate concentrations of 1500 mg/l (Templer *et al*., 2006) and lactose, by concentrations in the order of 350 mg/l (Lalman *et al*., 2004).

Besides the potential inhibition effect, LCFA accumulation onto the sludge can create a physical barrier and hinder the transfer of substrates and products (Pereira *et al.*, 2005).

In this study, sodium oleate was used as a model for long chain fatty acids because it is, in general, the most abundant of all LCFA present in wastewaters, has a good solubility and is considered one of the more toxic LCFA (Alves *et al.*, 2001a).

1.4 Biodegradability Assays

Anaerobic biodegradability assays are used to assess the ultimate methane potential of wastes, but are also used for determination of biodegradation rate in general.

Methods based on product formation measure either the gaseous end product (biogas) or liquid phase production of intermediates such as volatile fatty acids or others. Most methods in the literature are based on monitoring biogas production. Biogas production can be measured either through volume increase under constant pressure (volumetric methods), pressure increase in constant volume (manometric methods), or measurement of methane formation by gas chromatography. Gas chromatography is used to measure content of methane and carbon dioxide of the biogas that ends up in headspace of closed vials (Angelidaki *et al*, 2007).

Methods using volume or pressure for determination of biogas production in a closed vessel are inaccurate unless the methane content in the produced volume is monitored.

For a known organic compound, the theoretical methane yield potential can be calculated from the Buswell's equation (1.1).

$$C_nH_aO_b + \left(n - \frac{a}{4} - \frac{b}{2}\right)H_2O \rightarrow \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4}\right)CH_4 + \left(\frac{n}{2} - \frac{a}{8} + \frac{b}{4}\right)CO_2 \qquad (1.1)$$

However, in a reactor the practical yield obtained will always be lower due to several factors such as the use of the organic material to synthesize bacterial mass (typically, 5-10% of the organic material degraded). Under favorable conditions, with mainly water-

soluble matter, degrees of conversion up to 90-95% can be achieved. On the other hand, if the organic matter is highly particulate or structural such as manures, 30-60% conversion is normal (Angelidaki & Sanders, 2004).

Biodegradability assays can also be based on substrate depletion, where more complex analysis of specific compounds are used such as chemical analysis, spectrophotometry or gas chromatography. Substrate depletion can be determined either as non specific parameters such as volatile solids (VS), chemical oxygen demand (COD), dissolved organic carbon (DOC), or direct analysis of the specific compound being used as substrate (Angelidaki *et al*, 2007).

1.5 Microbial Community Analysis in Environmental Biotechnology

The microbial consortia catalyzing wastewater treatment have long been viewed as "black boxes". However, environmental biotechnology depends on the pollutant-degrading capacities of natural occurring microbial consortia (Watanabe & Baker, 2000; Amann *et al.*, 1998). Successful bioconversion will, thus, rely on the presence of a well balanced microbial community. Therefore, knowledge about the structure and function of mixed microbial communities present in bioreactors is essential for process optimization and process stability (Stams & Elferink, 1997; Amann *et al.*, 1998, Aoi, 2002).

Classical techniques for the identification of microorganisms, namely cultivation and simple light microscopic observation revealed to be insufficient to characterize complex microbial communities. Only a small fraction of the naturally existing microorganisms are possible to identify or detect using the conventional culture techniques. Moreover, media used in for the cultivation of microorganisms are selective giving a wrong idea of the real community composition (Embley *et al.,* 1996; Muyzer *et al.*, 1998; Watanabe & Baker, 2000; Yoshie *et al,*. 2001; Aoi, 2002; Calli *et al.,* 2005; Keyser *et al.*, 2006). Besides, the very limited morphology diversity among bacteria makes microscopic observation an insufficient mean to determine their identity (Amann *et al.*, 1998). Therefore, the microbial diversity becomes often underestimated.

Molecular biology tools have been largely applied to the study of microbial communities in different environments such as soil (Gelsomino *et al*., 1999; Torsvik & Øvreås, 2002, Watanabe *et al*., 2004), biofilms (Aoi, 2002; Cole *et al.*, 2002), wastewater treatment plants/bioreactors (Yoshie *et al.*, 2001; Calli *et al.* 2005, Buzzini *et al*., 2006; Keyser *et al.*, 2006) and aquatic (Fuhrman *et al*., 1993; Henriques *et al*., 2004;) have been reported in the literature.. Molecular biology methods are based in nucleic acids extraction from mixed microbial populations which are used in various approaches to determine the genetic diversity of the communities. As nucleic acids are extracted directly from a microbial consortium the cultivation bias is eliminated (Muyzer *et al.*, 1993; Watanabe & Baker, 2000).

The choice of 16S ribosomal RNA sequences

When the objective is to study the entire microbial community of a sample, and not only some specific metabolic groups within the community, usually the 16S rRNA gene is chosen because it occurs in all cellular organism, exhibit functional constancy, contain highly conserved and also highly variable regions. Most phylogenetic relationships can be measured because different positions in their sequences change at different rates and so, can be used as evolutionary biomarkers. Furthermore, in the last 20 years an explosive expansion of the SSU rRNA sequence database has been observed. (Embley & Stackebrandt, 1996; Watanabe & Baker, 2000).

Apart of the advantages, some disadvantages may be enumerated when using the 16S rRNA, like the heterogeneity between multiple copies within one specie which hampers pattern analysis and also the lack of resolution at the species level (Dahllöf, 2002).

DGGE – A PCR-based molecular tool for community fingerprint

Denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rDNA fragments combines a direct visualization of bacterial diversity and the opportunity of subsequent identification of community members by sequence analysis (Muyzer *et al.*, 1998). DGGE is now routinely used to assess the diversity of microbial communities and to monitor their dynamics, and it permits the simultaneous analysis of numerous samples.

The amplification of DNA extracted from mixed microbial communities with primers specific for 16S rRNA gene fragments of bacteria or archaea results in mixtures of PCR products of the same size. DNA fragments of the same length, but with different nucleotide sequences can be separated, on polyacrylamide gels, by electrophoresis through a linear gradient of increasing chemical denaturants of urea and formamide. The two strands of a DNA molecule separate or melt at a specific temperature, which depends on the hydrogen bonds formed between complementary base pairs (GC-rich domains melt at higher temperatures), and on the attraction between neighboring bases on the same strand. When running on a polyacrylamide gel, the mobility of the molecule is retarded when the first melting domain is reached, resulting in partial dissociation of the fragment. Complete strand separation is prevented by the presence of a high melting domain, the GC clamp (40-nucleotide GC-rich sequence), which is added to one of the PCR primers. Most of the DGGE studies focus on the number of different bands in order to get an estimate of the community richness (Watanabe & Baker, 2000; Schäfer & Muyzer, 2001; Dorigo *et al.*, 2005;).

The information collected by molecular tools reveal the complexity of whole bacterial communities but the interpretation of the observed variability is often ambiguous. However, there are nowadays available statistical tools available, by which a large amount of data can be analyzed. GelCompar software package is an example of computer-assisted guideline for the analysis of fingerprinting profiles. When considering the presence/absence of the bands, similarities between banding patterns, taken in pairs, can be expressed as a percentage value of a similarity coefficient such as Dice coefficient. Similarity or distance matrices can be displayed graphically as a dendrogram but also give way to clustering and ordination methods (Fromin *et al.*, 2002). The UPGMA is a

clustering method for binary data whereby pair-wise similarities of DGGE patterns are used to infer a dendrogram that depicts these distances in graphical form. A binary matrix is translated into a distance matrix representing the similarities of the DGGE patterns using a similarity coefficient. (Schäfer & Muyzer, 2001)

2 Anaerobic Biodegradation of Oleate. Assessment Before and After Degrading the Biomass Associated LCFA

2.1 Introduction

Accumulation of LCFA on anaerobic sludge was studied before in several works, during bioreactors operation, and the general conclusion pointed forward that sequencing accumulation and degradation steps was a strategic procedure to achieve an efficient rate of methane production as well as a stoichiometric methane yield. One of the most interesting results reported by Pereira *et al.* (2002), demonstrated that when feeding continuously oleic acid to an UASB reactor there was an accumulation of palmitic acid onto the sludge which was only biodegraded when oleic acid was not present in the medium. This clearly suggested that batch sequential operation would be the optimal strategy to treat LCFA rich wastewater. Recent results proved that the sequential operation is an essential step during start-up but, after few cycles, the sludge is able to efficiently convert continuously organic loading rates up to 20 kg $COD.m^{-3}.day^{-1}$ (50% COD as oleate), with methane conversion efficiencies higher than 80% (Cavaleiro *et al.*, 2007).

Several studies reported biodegradability and activity measurements of anaerobic sludge encapsulated with different amounts of LCFA (Pereira *et al.*, 2001; Pereira *et al.*, 2004). Also the kinetics of biodegradation of biomass associated LCFA was already established in a previous work (Pereira *et al.*, 2004). However, the behaviour of the sludge is highly dependent on the degree of acclimation to LCFA. A sludge acclimated to discontinuous or pulsed feeding of fat is probably the best inoculum to use for LCFA rich wastewater treatment, according to our previous results. Therefore, it was considered important to study a sludge previously acclimated to LCFA in those conditions.

In this chapter, a biomass highly loaded with LCFA, and acclimated as further explained, was studied before and after the degradation of the biomass-associated LCFA in terms of: (i) biodegradability of oleic acid in concentrations ranging from 100 to 1500 mg/l, and (ii) specific methanogenic activity in acetate and H_2/CO_2.

2.2 Materials and Methods

2.2.1 Biomass source

The suspended biomass used in this study was collected from a lab scale reactor fed with a synthetic dairy wastewater (made by dilution of skim milk with tap water) containing sodium oleate. The reactor was operated in continuous during 67 days, with an organic loading rate of 4.0 kg $COD.m^{-3}.day^{-1}$, at a constant temperature of 37 ± 1ºC during the trial period. Skim milk and oleate contributed each for 50 % of total COD fed. This substrate was supplemented with macro and micronutrients and 5 g $NaHCO_3$ were added per litre of feeding (Zehnder *et al.*, 1980). At the end of the operation the biomass was encapsulated by a whitish matter.

2.2.2 Biomass characterization

2.2.2.1 Specific methanogenic activity tests

Specific methanogenic activity, in the presence of acetate and H_2/CO_2, was determined before and after the degradation of the biomass associated LCFA.

Specific methanogenic activity tests were performed using a pressure transducer technique (Colleran *et al.*, 1992; Coates *et al.*, 1996). The test involves the monitoring of the pressure increase developed in seal vials fed with non-gaseous substrates (acetate) or pressure decrease in vials previously pressurized with gaseous substrates (H_2/CO_2). The hand-held pressure transducer (Centrepoints Electronics, Galway, Ireland) was capable of measuring a pressure increase or decrease of 2 bar (0 to ± 202.6 kPa) over a range of -200 to +200 mV, with a minimum detectable variation of 0.005 bar. A volume of approximately 30 µl of biogas is released by each pressure measurement, which represents an insignificant fraction of the total biogas produced during the test. Blank controls were used for liquid substrates (no added substrate) and for gaseous substrates (pressurized with N_2/CO_2). The basal medium used in the batch experiments was made up with

demineralised water and was composed of cysteine-HCl (0.5 g/l), resazurin (1 ml/l) and sodium bicarbonate (3 g/l), the pH was adjusted to 7.0-7.2 with NaOH 8 N and was prepared under strict anaerobic conditions. No calcium or trace-nutrients were added. Methanogenic activity tests were carried out in 25 ml vials when using acetate and 70 ml vials when using gaseous substrates. In both cases, the working volume was 12.5 ml and the VS concentration was in the range of 2 to 5 g/l. Biomass was washed and centrifuged (4000 rpm, 10 min, 4ºC) twice with basal medium, dispensed in the vials with an appropriate amount of basal medium and incubated overnight (37ºC, 150 rpm), for temperature acclimation. After, the excess pressure was vented and the substrates were added. A volume of 0.125 ml of acetate was dispensed from stock solutions 100 fold concentrated, in order to obtain in the test vial concentrations of 30 mM. For gaseous substrates, H_2/CO_2 80/20 (vol/vol) and N_2/CO_2 80/20 (vol/vol) were pressurized at 1 bar in the test and blank vials, respectively. At the end of the assay, the methane content of the biogas, produced in the test and blank vials of liquid substrates, was determined by gas chromatography using a Porapack Q (100–180 mesh) column, with He as carrier gas at 30 ml/min and a thermal conductivity detector. Temperatures of the injection port, column and detector were 110, 35 and 110ºC, respectively. Methane quantification was made by comparison of the sample peak area with the one obtained after injection of a standard (mixture of CH_4 (40%), CO_2 (40%) and N_2 (the remaining)). For all the vials, the headspace volume was individually determined by recording the pressure increase when a known volume of air was injected in the closed vial (mV/ml). This headspace correction factor (mV/ml) is used to convert recorded millivolt readings to ml of biogas produced. The VS content of each vial was determined according to Standard Methods (1989). All the batch experiments were performed in duplicate assays.

Calculation of methanogenic acetoclastic activity

The percentage of methane (MP) in the biogas produced in each vial during the test was determined as shown in equation 2.1.

$$MP = \frac{(Vh + Vc)}{Vc \times \%CH4} \quad (2.1)$$

where,

Vh = volume of headspace (ml)

Vc = volume of biogas produced during the test (ml)

$\%CH_4$ = percentage of methane in the biogas determined in the end of the assay

Vc and Vh are calculated by equations 2.2 and 2.3, respectively.

$$Vc = \frac{\text{final mV reading (mV)}}{\text{headspace correction factor (mV/ml)}} \quad (2.2)$$

$$Vh = \frac{\text{mV reading at 1 atm relative pressure}}{\text{headspace correction factor (mV/ml)}} \quad (2.3)$$

The specific methanogenic activity (SMA) is determined according to equation 2.4 and expressed as $mlCH_{4(STP)}.gVS^{-1}.day^{-1}$.

$$SMA = \frac{GP \times MP \times CF}{VS} \quad (2.4)$$

where,

GP = volume of biogas produced per day, determined by equation 2.5

CF = calibration factor to convert to Standard Temperature and Pressure conditions (STP), determined by equation 2.6

VS = volatile solids in each vial

$$GP = \frac{\text{initial slope of the plot mV readings vs time (mV/h)}}{\text{headspace correction factor (mV/ml)}} \times 24 \qquad (2.5)$$

$$CF = \frac{100}{\text{mV reading at 1 atm}} \times \frac{273}{273 + 37} \qquad (2.6)$$

Calculation of methanogenic hydrogenotrophic activity

The conversion of H_2/CO_2 to CH_4 is accompanied by a decrease in gas volume and a reduction of headspace pressure in the test vials, which corresponds to the transformation of 4 moles of H_2 and 1 mol of CO_2 into 1 mole of methane, according to equation 2.7.

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad (2.7)$$

The pressure decrease was directly converted to ml of methane produced by equation 2.8.

$$\text{volume } CH_4 \text{ (ml)} = \frac{P(n-1) - P(n)}{mV/ml \times 4} \qquad (2.8)$$

where,

$P_{(n-1)}$ = mV reading at time n-1

$P_{(n)}$ = mV reading at time n

mV/ml = headspace correction factor

The cumulative methane production is calculated by addition of the CH_4 volumes produced between each reading. The initial slope of the methane production curve (ml CH_4/h) was determined and the specific methanogenic activity was calculated according to equation 2.9. and expressed as ml $CH_{4(STP)}/gVS^{-1}.day^{-1}$).

$$SMA = \frac{\text{ml } CH_4/h \times 24 \times CF}{VS} \qquad (2.9)$$

Specific methanogenic activity values were converted to mg $COD\text{-}CH_4/gVS^{-1}.day^{-1}$) (350 ml CH_4 are theoretically produced for each g of COD) and background methane production due to the residual substrate (blank controls) was discounted.

2.2.2.2 Extraction and GC analysis of LCFA accumulated onto the biomass

An aliquot of LCFA-containing biomass was transferred into glass tubes and dried at 85ºC. Dry weight was determined and, after, a solution of internal standard (1.5 ml), dichloromethane (2 ml), Milli-Q water (2 ml) and a HCl:1-propanol solution (1.5 ml) were added to the dry sample. The internal standard used was pentadecanoic acid (C15) and the composition of all the solution used is described in Appendix A. After digestion during 3.5 h at 100ºC (closed reflux) the content of each vial was transferred with Milli-Q water to glass vials, immediately capped, with rubber septa and aluminium crimp caps, and were maintained in inverted position for 20 minutes. The dichloromethane phase was then transferred to glass vials and a small quantity of solid sodium thiosulfate ($Na_2S_2O_3$) was added to guarantee that samples were dehydrated. The vials were immediately capped and stored at 4ºC, until chromatographic analysis. The extraction of LCFA from biomass samples was performed in duplicate.

In this case, LCFA were extracted from biomass samples with dichloromethane and submitted to a chemical derivatization, more precisely a propylation (equation (2.10)).

$$RCO_2H + CH_3CH_2CH_2OH \xrightarrow{H^+} RCOOCH_2CH_2CH_3 + H_2O \qquad (2.10)$$

Esterified LCFA were separated by gas chromatography (Chrompack CP 9001), with a TR-WAX (eq.CP-Sil 52 CB) 30 m × 0.32 mm × 0.25 μm capillary column connected to a flame ionization detector and to a split/splitless injector (split ratio 1:10). Helium was the gas carrier at a flow rate of 1 ml/min, H_2 (30 ml/min) was the combustion gas and N_2 (30 ml/min) was the make-up gas. The column, injector and detector temperatures were maintained at 50 to 225ºC (10ºC/min), 220ºC and 250ºC respectively.

Calibration curves were prepared with lauric (C12), myristic (C14), palmitic (C16), palmitoleic (C16:1), stearic (C18), oleic (C18:1) and linoleic (C18:2) acids, and the calibration procedure is described in Appendix A.

2.2.3 Oleate biodegradability before and after degradation of biomass associated LCFA

Oleate biodegradability capacity of this biomass was studied before and after degradation of the biomass associated LCFA.

A set of 20 vials was prepared as described in section 2.2.2.1. After an overnight incubation (37ºC, 150 rpm), a volume of 1.25 ml of oleate stock solutions, 10 fold concentrated, was added to 8 of these vials, in order to obtain the following concentrations inside the vials: 100, 500, 1000 and 1500 mg/l (duplicate assays). Other 8 vials, received the same amount of substrate, which was added after the stabilization of the biogas production due to the degradation of the biomass associated LCFA. Before oleate addition, the vials were vented and the headspace washed for approximately 10 seconds with N_2/CO_2 (80:20). These tests were performed in duplicate. A blank control assay (without oleate addition) was performed in parallel, corresponding to the remaining 4 vials. From the moment of substrate addition, the methane production was determined by measuring periodically pressure and methane content of the biogas accumulated in the headspace, until stabilization.

2.3 Results and Discussion

The sludge analyzed in this chapter was sampled from a lab-scale reactor, operated as described in the Materials and Methods (section 2.2.1). During the reactor operation the methane production was significantly reduced and the biomass became surrounded by a whitish matter, which was expected to be palmitic acid as was observed in previous works (Pereira *et al.*, 2002). Extraction and GC analysis of individual LCFA between C12:0 and C18:2, confirmed that palmitate represented 91% of the total LCFA accumulated onto the sludge, and also traces of myristate and oleate were detected (Table 2.1).

Table 2.1 Long chain fatty acids associated to the biomass before the degradation of the accumulated substrate, in the beginning of the assay (mean ± SD).

	Oleic acid	**Palmitic acid**	**Myristic acid**
mg LCFA. g TS^{-1}	12 ± 0.4	268 ± 9	14 ± 0.4
% of each LCFA	4	91	5

Figure 2.1 (a) and (b) presents the cumulative methane production obtained in the biodegradability experiment performed before and after the degradation of the biomass-associated substrate, respectively, and Table 2.2 summarizes the percentage of methane-COD recovered in both experiments.

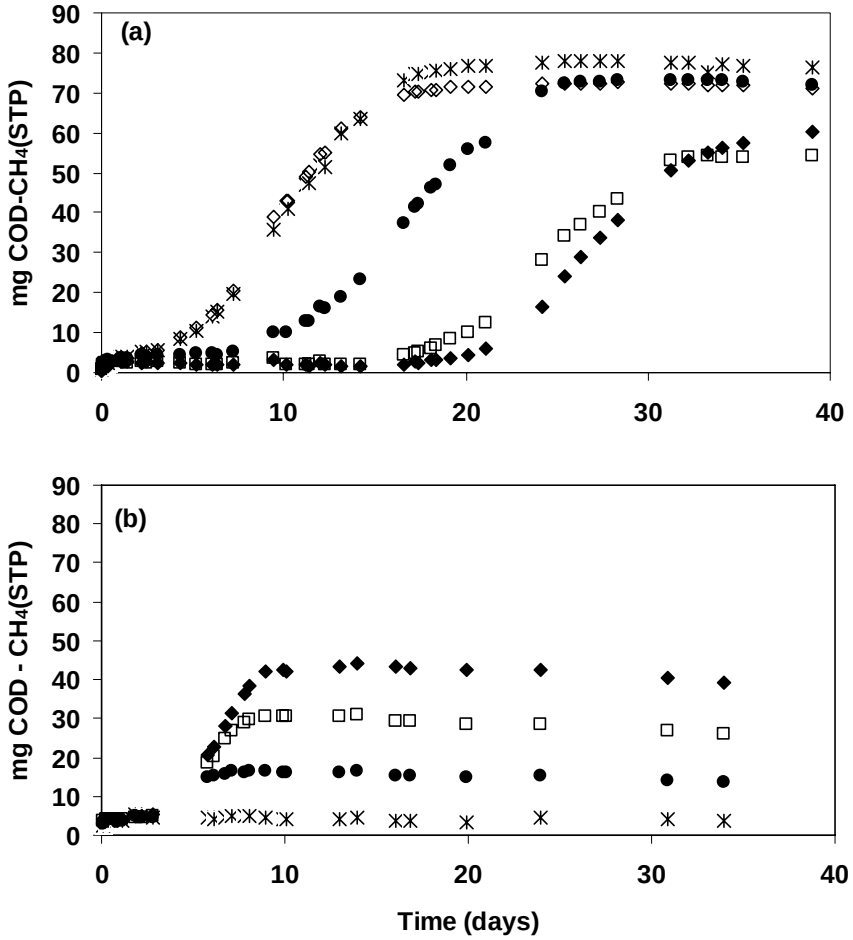


Figure 2.1 Anaerobic biodegradation of oleate (a) before and (b) after the degradation of the biomass-associated substrate. ◊ - no oleate added; ✳ – 100 mg/l oleate; ● – 500 mg/l oleate; □ – 1000 mg/l oleate; ♦ - 1500 mg/l oleate.

When the encapsulated biomass was placed in batch vials, without any added substrate, it was capable of degrading the adsorbed substrate (Figure 2.1 (a) - ◊ - no oleate added). The onset of methane production was observed after three days of incubation, suggesting some difficulties on substrate degradation or in the methane formation/release to the headspace. The addition of increasing oleate concentration resulted in a proportional increase of the lag phase preceding the initial methane production, except when 100 mg/l of oleate were added**.** In this case, methane production presented the same behaviour of the blank assay (in which methane production resulted from the degradation of the accumulated substrate) during the first 16 days. After this period, methane production in

the blank assay stopped and in the 100 mg/l vials continued, reaching a plateau of 78 mg COD-CH_4 instead of the 73 mg COD-CH_4 obtained in the blank assay (Table 2.2).

Table 2.2 Percentage of COD-CH_4 recovered before and after the degradation of biomass associated LCFA, in the presence of different concentrations of oleate.

	Before degrading the biomass associated LCFA			After degrading the biomass associated LCFA		
Test Concentration (mg/l)	Initial COD (mg COD)	Methane produced (mg COD)	% COD – CH_4 recovered	Initial COD (mg COD)	Methane produced (mg COD)	% COD – CH_4 recovered
0	73 ± 3 (*)	73 ± 3		2.64 ± 0.04 (*)	2.64 ± 0.04	
100	76	78 ± 4	102.5 ± 5.8	7	5.1 ± 0.2	81.0 ± 3.6
500	91	73 ± 5	80.9 ± 6.1	22	16.9 ± 1.1	81.7 ± 5.4
1000	109	55 ± 0.1	50.9 ± 1.2	40	30.8 ± 0.1	79.4 ± 0.2
1500	127	62 ± 6	48.6 ± 4.9	58	44.0 ± 2.6	77.5 ± 4.6

(*) indirectly estimated by the plateau in the cumulative methane production curve ("blank assay")

When 500 mg/l were added, the same plateau of the blank assay was reached after a lag phase of 7 days, but when higher concentrations were present a lower plateau was observed in the time course of the experiment. In all cases, except for the vials where 100 mg/l oleate were added, the theoretical methane production from the degradation of the oleate added, in addition to the accumulated substrate was never achieved (Table 2.2). In the presence of 1000 and 1500 mg/l the sludge was able to convert to methane only about 50% of the total substrate present in the vials, and a lag phase of 17 days before methane production started was observed. These results show that oleate addition influenced the delay observed before methane production started and also the maximum plateau reached in each vial. These effects may be due to mass transfer limitations (Pereira *et al.*, 2005), inhibition or a combination of both phenomena. In this work, no effect was caused by a concentration as low as 100 mg/l, but a moderate effect was observed with 500 mg/l of oleate and a more evident one was caused by the highest concentrations (1000 and 1500 mg/l). Other authors have reported evident differences between blank assay and the assay in which 100 mg/l were added, showing that this oleate concentration was clearly inhibitory, either in terms of methane production rate or in terms of plateau obtained

(Pereira *et al.*,2002; Lalman & Bagley, 2002). Also, Perle *et al.* (1995) reported that in anaerobic treatment of dairy effluents the milk fat concentration should be lower than 100 mg/l (Perle *et al*., 1995).

After degrading the associated LCFA, the biomass oleate biodegradation capacity was enhanced, since the conversion of oleate to methane was in the range of 78 and 82%, for all the concentrations tested (Table 2.2). Lag phases (during approximately 3 days) were considerably smaller than the ones observed previously.

From the assay performed without any added oleate (blank assay), it is possible to indirectly estimate the amount of biomass-associated substrate from the plateau achieved in the cumulative methane production curve. The obtained value per gram of volatile solid measured at the end of the assay was 5985±600 mg COD-CH4.g VS^{-1}. The initial methane production rate from the accumulated substrate was 146 ± 10 mg COD-CH4.gVS^{-1}.day^{-1}. This sludge was more loaded that the one referred previously by Pereira *et al.* (2005) which accumulated 4.6 g COD-LCFA.g VS^{-1} when fed with pure oleic acid at an organic loading rate of 6 kg COD.m^{-3}.day^{-1}. In that case, the accumulated substrate was mainly composed of LCFA, 83% as palmitic acid. However, it is reasonable to admit that, in the present work, the substrate accumulated in the sludge was also composed of other components present in the co-substrate (skim milk). For instance complexes of proteins and LCFA could be entrapped in the biological aggregates. This conclusion can be withdrawn by comparing the value of 5985 ± 600 mg COD-CH4.g VS^{-1}, obtained from the plateau of the cumulative methane production curve, with the analysis of the most dominant LCFA expressed as mg COD-LCFA per gram of total dry weight (Table 2.1). The comparison between those values should consider the difference between the meaning of VS and TS. VS represent the volatile solids concentration, corresponding to the cells, at the end of the "blank assay", that means, after degrading virtually all the accumulated substrate. However, in the analysis of LCFA content, the sample is initially dried and therefore in this case, TS represents the sum of the initial mass of cells plus the substrate. In the present experiment, the sludge was loaded with a complex substrate containing oleate which is a more realistic approach than the strategy followed in some previous tests where oleate was the sole organic carbon source fed to the reactors. In fact, in a real

situation i.e. for instance in a dairy wastewater, proteins and carbohydrates are also present.

Table 2.3 presents the specific methanogenic activity in the presence of acetate and H_2/CO_2, measured before and after the degradation of the accumulated substrate. The extremely high value of substrate associated to the sludge by mechanisms of adsorption, precipitation and/or entrapment did not inhibit completely the specific methanogenic acetoclastic activity, as was previously observed for sludges loaded with amount of substrate in the range of 2.8 to 4,5 g COD.g VS^{-1} (Pereira *et al.,* 2004).

Table 2.3 Specific methanogenic activities exhibited by the biomass, before and after the degradation of the accumulated substrate (mean ± SD).

SMA in the presence of:	**(mg COD-CH_4.g VS^{-1}.day^{-1})**	
	Before substrate degradation	**After substrate degradation**
Acetate	148 ± 39	539 ± 55
H_2/CO_2	1416 ± 90	2970 ± 327

The specific methanogenic activity, in the presence of acetate and H_2/CO_2, of the encapsulated sludge was 148 ± 39 and 1416 ± 90 mg COD-CH_4. g VS^{-1}.day^{-1}, respectively (Table 2.3). After degrading the associated LCFA the biomass SMA increased about 2 times in the presence of H_2/CO_2 and approximately 4 times in the presence of acetate (Table 2.3). In a previous work made by Pereira *et al.* (2004) three sludges with different loads of biomass-associated substrate were characterized in terms of SMA before and after its degradation. Figure 2.2 represents the influence of the amount of substrate accumulated onto the sludge on the specific methanogenic activity measured after its degradation. A trend towards a maximum specific acetoclastic activity of about 550 mg COD-CH4.gVS^{-1}.day^{-1} and a maximum specific hydrogenotrophic activity of about 2800 mg COD-CH4.gVS^{-1}.day^{-1} is evident, suggesting that heavy accumulation, followed by degradation of complex substrates with high lipid content, is beneficial to develop an efficient methanogenic consortium. Furthermore it is relevant to note that, despite the huge amount of accumulated substrate and LCFA that were in contact with the sludges, both trophic

groups, hydrogen and acetate consumers, showed a similar pattern of SMA increasing, which is particularly interesting since the acetoclastic group is usually reported as more sensitive to LCFA toxicity than hydrogenotrophic group (Hanaki *et al.*, 1981). The hypothesis already proposed by Pereira *et al.* (2005) about the important role of mass transfer limitations in LCFA degradation processes, is likely the reason for the reported "high toxicity" of LCFA towards acetoclastic bacteria. Since acetate is a larger molecule, when comparing with H_2, is likely more difficult to be transported through a layer of LCFA surrounding the cells in an encapsulated sludge.

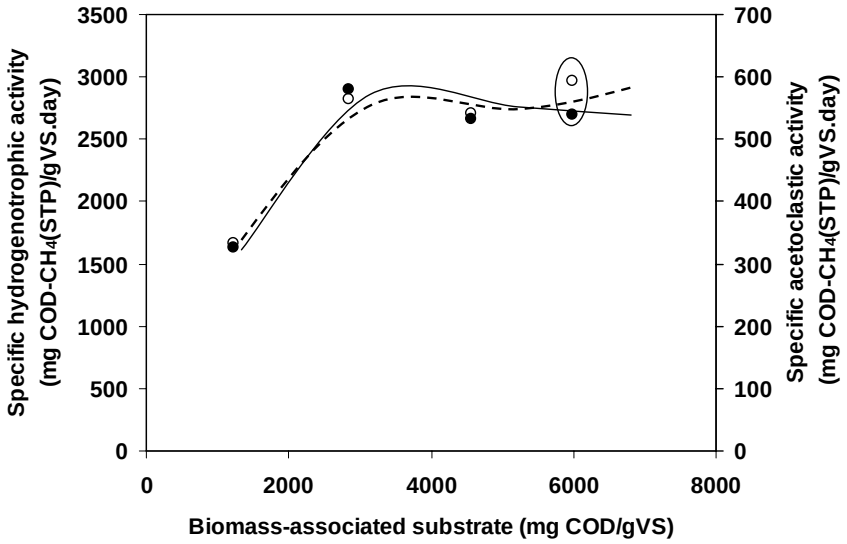


Figure 2.2 Influence of the amount of substrate accumulated onto the biomass, in the specific methanogenic activity in (•) acetate and (o) H_2/CO_2 after its degradation. The marked points represent the values obtained in the present work. The other three points were obtained by Pereira *et al*., 2004, where the biomass-associated substrate was only composed of LCFA.

The existing literature suggests that LCFA cause an irreversible toxic effect to the biomass. However, this idea was contradicted by Pereira *et al.* (2003b, 2004, 2005) and the results obtained in this work confirm that the inhibitory effect caused by oleic acid is reversible, even when concentrations in the order of 1500 mg/l are applied and when the biomass was severely loaded with LCFA. Because LCFA degradation depends on the interaction of different metabolic groups, which act in syntrophy, the loss of activity of a group, even if it is temporary, influences negatively the overall process, causing a delay on

the degradation of LCFA and consequently on methane production. Therefore, besides SMA, it is also important to study the capacity of this sludge to biodegrade the accumulated substrate and compare it with previously studied sludges.

Pereira *et al.* (2004) studied the influence of the available accumulated substrate on the kinetics of its own mineralization and suggested that a simple inhibition model based on the Haldane substrate inhibition enzymatic kinetics was not able to describe the kinetics of mineralization of the biomass associated LCFA, likely because other factors such as mass transfer limitations and the degree of sludge acclimatization were not taken into account. Furthermore, the method used to measure the amount of the accumulated substrate is based on the measurement of the final product (methane) and thus depends on the metabolic activity of different trophic groups present in the consortium. Figure 2.3 presents the experimental points resulted from other studies (Pereira *et al.*, 2004), as well as a new point corresponding to the methane production rate obtained from the degradation of the adsorbed LCFA by the biomass used in the present study.

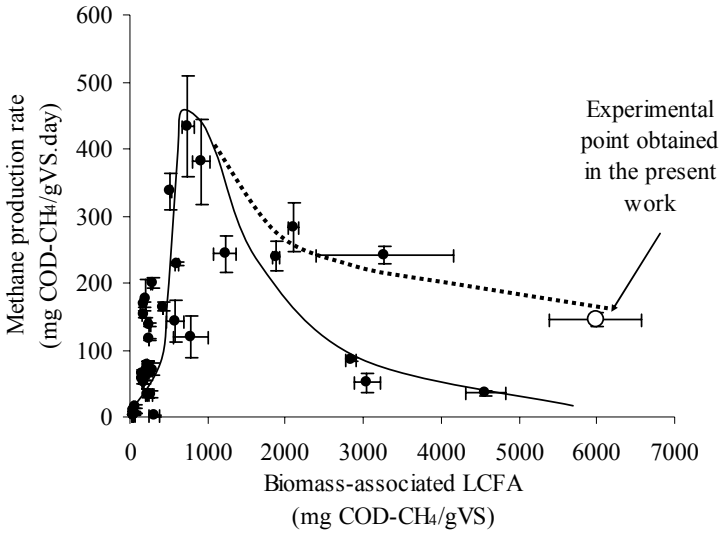


Figure 2.3 Methane production in batch assays due to the degradation of the biomass associated LCFA. Two curves of tendency are suggested: (—) corresponding to less acclimated sludges and (- - -) to more acclimated sludges. Bars represent the standard deviation.

It seems that two different tendency curves can be adjusted to the experimental data, probably one corresponding to data obtained with sludges which were acclimated and other to data obtained with less acclimated sludges (Figure 2.3).

3 Anaerobic Biodegradation of Oleate. Analysis of Intermediates

3.1 Introduction

LCFA are biodegraded, through β–oxidation, after transport into the bacterial cell. Direct β–oxidation of oleic acid would produce all the possible β–oxidation byproducts until acetate, including palmitoleic (C16:1), an unsaturated LCFA. On the other hand, some authors propose that a hydrogenation occurs before oleate biodegradation (Lalman & Bagley, 2000). In this case, only saturated intermediates would be expected, namely stearic (C18), palmitic (C16), myristic (C14), lauric (C12) and other fatty acids shorter by two carbon atoms until acetate.

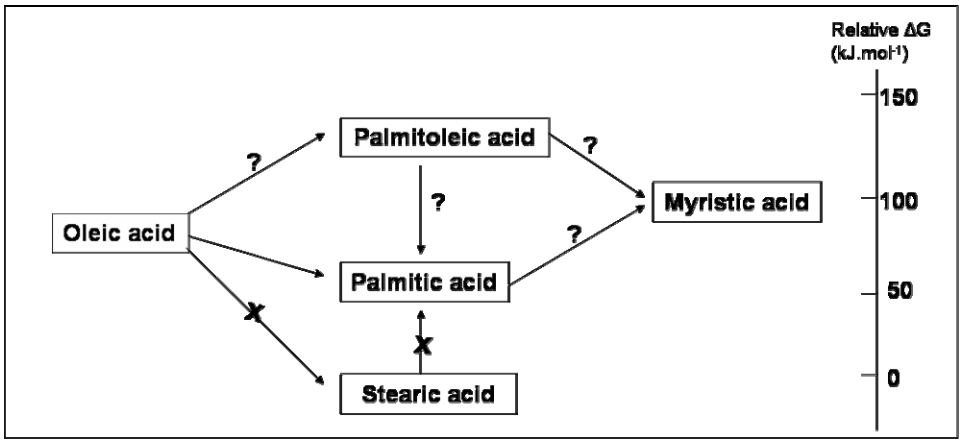
The inability to detect all β–oxidation intermediates, in the liquid medium or even in association to the biomass, is not unexpected since the reactions occur within the cells. The detection of intermediates may indicate a saturation of the organisms' capability to process the intermediates (Lalman & Bagley, 2000) or a preference for one substrate instead of another.

In the literature, some of the predictable LCFA intermediates of oleate degradation were never detected. Lalman and Bagley (2001) studied the degradation of oleic acid under anaerobic conditions at 21ºC, and during 30 days oleate was not detected in the liquid medium. However, palmitic and myristic acids as well as trace amounts of acetic acid were observed, but stearic was not detected, although the conversion of oleate to stearate is energetically more favored than the conversion to palmitic or palmitoleic (Table 3.1).

Table 3.1 Free energy values for oleate conversion reactions. (Adapted from Lalman & Bagley, 2001)

Reactant → Product	**Reactions**	**$\Delta G^{0'}$ (kJ.mol^{-1})**
Oleic acid → Stearic acid	$C_{18}H_{33}O_2 + H_2 \rightarrow C_{18}H_{35}O_2$	-78.6
Oleic acid → Palmitic acid	$C_{18}H_{33}O_2 + 2H_2O \rightarrow C_{16}H_{31}O_2 + C_2H_3O_2 + H_2 + H^+$	-27.8
Oleic acid → Palmitoleic acid	$C_{18}H_{33}O_2 + 2H_2O \rightarrow C_{16}H_{29}O_2 + C_2H_3O_2 + 2H_2 + H^+$	50.5
Stearic acid → Palmitic acid	$C_{18}H_{35}O_2 + 2H_2O \rightarrow C_{16}H_{31}O_2 + C_2H_3O_2 + 2H_2 + H^+$	50.8

The free energies presented in Table 3.1 were calculated at standard conditions of 25ºC, 1 atm and activities of 1, except for hydrogen ion which has been corrected to an activity of 10^{-7} (pH = 7) (Lalman & Bagley, 2001). Stearate was not detected probably because energy is required for β-oxidation to palmitic acid (Table 3.1 and Figure 3.1). The products of oleate degradation are shown in Figure 3.1 on the basis of their relative reaction energies. The products at the end of arrows marked with ***?*** have been detected but the pathway is either energetically unfavorable under standard conditions or otherwise uncertain. The products at the end of the arrows marked with ***X*** have not been detected experimentally, although they may be energetically favorable (Lalman & Bagley, 2001).



X = not detected ? = product detected, pathway uncertain

Figure 3.1 Degradation pathways for oleic acid. (adapted from Lalman & Bagley, 2001)

Several studies have been made about oleate anaerobic degradation, most of them concerning the inhibition of anaerobic microorganisms by this kind of organic compound (Koster & Cramer, 1987; Lalman & Bagley, 2000; Vidal *et al*., 2000; Templer *et al*., 2006). The distribution of the intermediates of oleate degradation during complete oleate conversion to methane has never been studied. In this chapter, the distribution of oleate and oleate degradation intermediates between the liquid phase and the biomass (solid phase) was studied in batch assays, with two consortia, one acclimated to LCFA and other unacclimated. Both, LCFA and VFA intermediates were monitored. Methane production was also monitored during the time course of oleate degradation.

3.2 Materials and Methods

3.2.1 Biomass sources

Both sludges were obtained from a local municipal sludge digester (Freixo, Porto, Portugal). Biomass 1 was used without prior acclimation to LCFA and Biomass 2, considered to be acclimated, was originally sampled from the same digester, but was in contact afterwards with oleic acid in a mesophilic lab scale reactor, that was fed with a synthetic dairy wastewater containing sodium oleate at organic loading rates between 2.5 and 5 kg $COD.m^{-3}.day^{-1}$ during around 50 days. Substrate composition was the same as described in section 2.2.1.

3.2.2 Biomass characterization

The two sludges were characterized in terms of specific methanogenic activity as previously explained in section 2.2.2.1. The biomass associated LCFA were analyzed as described in section 2.2.2.2.

3.2.3 Batch assays

For each biomass a set of 28 vials was prepared as described in section 2.2.2.1, with the exception of the type of substrate added. In 26 vials, a volume of 1.25 ml of oleate stock solution was added, in order to obtain a final COD-oleate amount of 30 mg (in a working volume of 12.5 ml). Methane production was monitored in the remaining two vials where no oleate was added (blank controls) as well as in two of the 26 vials where oleate was added.

At certain moments, vials were sacrificed in duplicate. The total content was transferred to centrifuge tubes and centrifuged (10 min at 4000 rpm, 4ºC), in order to separate the solid phase (biomass + biomass-associated LCFA) from the liquid medium. LCFA concentration was determined in the solid phase (associated to the biomass) by gas

chromatography as previously described in section 2.2.2.2. The same methodology was applied for LCFA quantification in the liquid phase with the exception of the first drying step, and with addition of 2 ml of sample instead of 2 ml of Milli-Q water. Volatile fatty acids were also analyzed in the liquid phase by HPLC (Jasco, Japan) using a Chrompack organic analysis column (6.5 × 30 mm) and a mobile phase of sulfuric acid (0.01 N) at a flow rate of 0.7 ml/min. The column temperature was set at 60ºC and the detection was made spectrophotometrically at a wavelength of 210 nm. Samples were centrifuged (10 min at 15000 rpm), filtered (0.2 μm) and stored at -20ºC until analysis. Calibration curves were prepared with formic, acetic, propionic, iso-butyric, n-butyric and valeric acids with concentrations ranging from 25 to 2500 mg/l.

LCFA and VFA concentrations were calculated in each sacrificed vial and values were converted to mg COD-LCFA and mg COD-VFA, respectively.

3.3 Results and Discussion

In general, the behaviour of the two sludges was not considerably different (Figure 3.2 (a, b)).

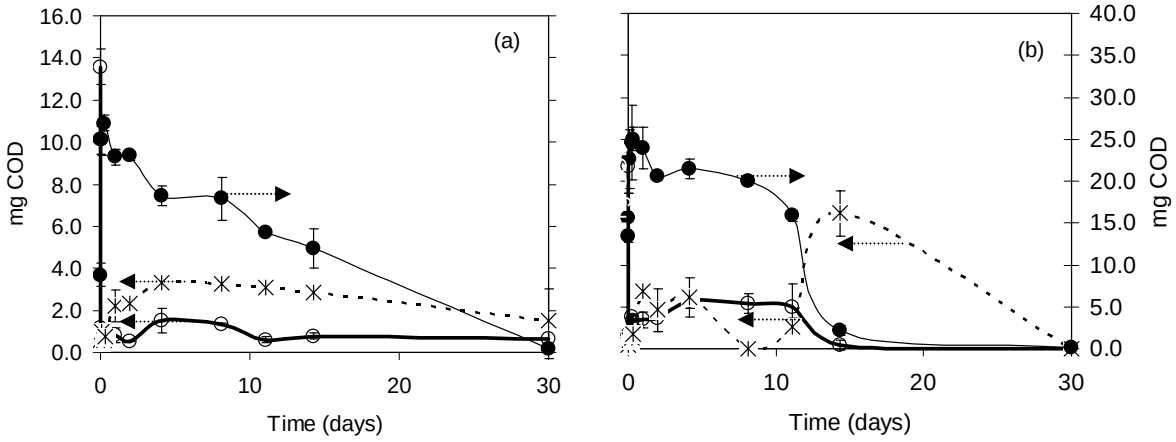


Figure 3.2 Time course of COD-oleate in the liquid (o), in association to the biomass (•) and COD - acetate (∗) during the degradation of oleic acid. (a) Non acclimated Biomass 1. (b) Acclimated Biomass 2.

For Biomass 1, 96 % of the initially added oleic acid adsorbed onto the solid phase in the first 50 minutes and for Biomass 2 the percentage of adsorbed oleate was 98%. After this moment, no LCFA were detected in quantities higher than 1.5 mg COD (Biomass 1) and 2.3 mg COD (Biomass 2), in the liquid phase. The pattern of oleic acid degradation and acetate accumulation was different for the two sludges. In the non acclimated sludge, the oleic acid quantity decreased progressively at an approximately constant rate, until day 30, when a residual amount of 1.5 mg COD-oleic acid was detected. Acetate increased up to 3.3 mg COD in the first four days and decreased afterwards accompanying the oleate decrease. At the end, a residual acetate amount of 1.5 mg COD was still detected. In the case of the acclimated sludge, oleic acid degradation was slower in the first 10 days, but a sharp decrease of oleate quantity was observed around day 12, followed by a peak of 6.5 mg COD-acetate on day 14, preceding the complete consumption of acetate, which was not detected at the end of the experiment. For both assays, acetic acid was the only volatile fatty acid detected.

In both assays, other LCFA, saturated and unsaturated, with 12 to 18 carbon atoms, except myristic acid (C_{14}), were also detected in residual quantities in the solid phase. Palmitic acid was the most dominant LCFA intermediate and was detected in all samples analyzed, although not exceeding a quantity of 0.75 mg COD (data not shown).

These results do not completely agree with the ones obtained by Petruy & Lettinga (1997) who reported that, during the operation of an EGSB reactor treating a milk fat emulsion, 70% of the lipids was adsorbed onto the granular sludge within approximately one day and that only the remaining lipids were converted slowly to methane gas during 26 days of operation. In this work it was evident that substrate which adsorbed (oleate) is the one degraded, since small amounts of oleate were found in the liquid medium after the first hour and almost all substrate was degraded during 30 days (Figure 3.2 and 3.3). Similar results were obtained by Hwu *et al.* (1998b) who studied the biosorption of LCFA in UASB treatment process and verified that 80% of the lipids adsorbed onto the sludge within 20 min. These authors reported that the biosorption of oleate was followed by a desorption and a subsequent adsorption to the surface of sludge granules and that after the ultimate adsorption, biodegradation proceeded during 40 days, although a complete biodegradation was only achieved at an oleate concentration of 300 mg/l. In this study, and according to the observed in Figure 3.2 (a, b), there was also an oleic acid desorption

detected between days 2 and 4 for both sludges. However, in the present study a complete degradation of oleic acid at a concentration of 830 mg/l was observed.

Figure 3.3 (a, b) represents the time course of the sum of all VFA+LCFA detected (mg COD) and also the cumulative methane production during 30 days, after oleate addition. In both assays about 30 mg COD-CH4 were produced.

Lag phases of approximately 12 and 10 days were observed for Biomass 1 and 2, respectively, before the onset of methane production. Oleate mineralization stopped after 12 days with biomass 1, whereas biomass 2 only needed 5 days to complete oleate mineralization.

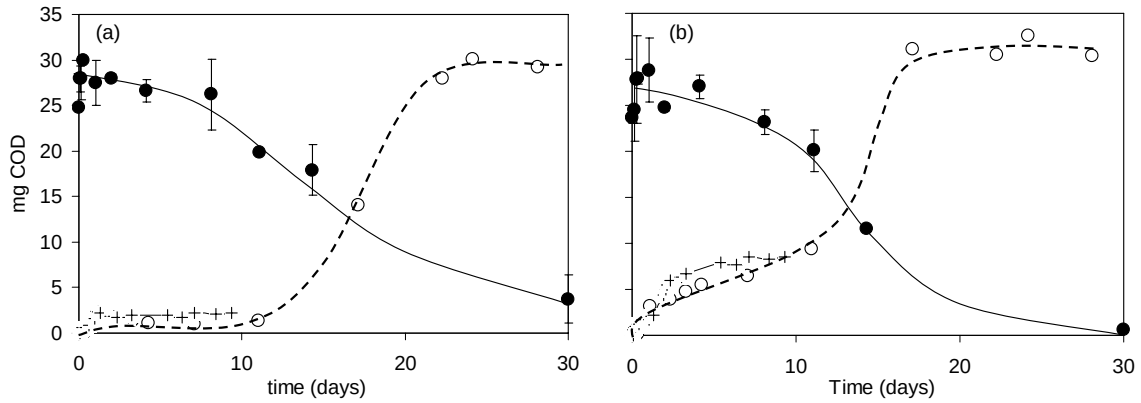


Figure 3.3 Time course of the cumulative methane production (o) and depletion of LCFA+acetate (•) in the unacclimated Biomass 1(a) and in the acclimated Biomass 2 (b). In both graphs the methane production in the blank assays are also presented (+).

The methane production observed during the lag phases corresponds to the production occurring in the blank vials, in which no oleic acid was added (Figure 3.3 (+)). This production is the result of the degradation, and consequent conversion to methane, of compounds associated to the biomass. In the blank assays, about 2 and 8 mg COD-CH_4 were formed from the residual substrate associated to Biomasses 1 and 2 respectively. However, only 0.2 mg COD-LCFA (Biomass 1) and 0.4 mg COD-LCFA (Biomass 2) associated to the biomass were quantified (Table 3.2) which means that other compounds, not identified in this study, may be present and were preferentially degraded.

Table 3.2 Long chain fatty acids associated to each biomass (mean ± SD).

	Biomass 1	**Biomass 2**	
	Oleic acid	**Palmitic acid**	**Oleic acid**
mg LCFA. g TS^{-1}	1.8 ± 0.09	1.8	1.3
mg COD in each vial	0.2 ± 0.012	0.2	0.2

The SMA exhibited by the two biomasses was different (Table 3.3). Even though none of the consortia had high methanogenic activity in the presence of acetate and H_2/CO_2, acetoclastic activity for Biomass 1 was not detected. Considering the fact that both sludges have the same origin, but that sludge 2 was acclimated by a previous contact with oleic acid in a fed batch reactor, it can be concluded that acclimation was beneficial to promote the acetoclastic activity and a better performance in terms of oleic acid degradation.

Table 3.3 Specific methanogenic activities exhibited by Biomass 1 and Biomass 2 (mean ± SD).

SMA in the presence of:	**(mg COD-CH_4.g VS^{-1}.day^{-1})**	
	Biomass 1	**Biomass 2**
Acetate	0	72 ± 10
H_2/CO_2	148 ± 27	118 ± 4

The accumulation of palmitic acid during LCFA rich wastewaters treatment in continuous anaerobic bioreactors is normally observed (Pereira *et al.*, 2002a). In batch vials, palmitic acid accumulation was previously reported by Cavaleiro *et al.* (2005), but in the present work there was only a residual accumulation of this LCFA. The behavior in continuous and in batch conditions is not comparable and the continuous feeding of oleic acid to a sludge encapsulated with palmitic acid seems to be inhibitory (Pereira *et al.*, 2004). The detection of intermediates of LCFA degradation accumulated in the solid phase was expected to give more insights onto the mechanisms of oleic acid degradation. However, there was not significant amount of LCFA detected in the present experiment, probably because the oleic acid, in the tested concentration, was progressively converted

into intermediates, which were then rapidly converted to methane. In this respect, it would be interesting to study higher oleate concentrations and also to follow the intermediates accumulated in the solid phase during the mineralization of biomass associated LCFA of a heavily loaded sludge, similar to the sludge studied in Chapter 2.

4 Anaerobic Microbial Community Dynamics During Discontinuous Operation of a Reactor Treating a Synthetic Dairy Wastewater

4.1 Introduction

Little is known about the microbial communities developed in anaerobic bioreactors treating LCFA rich wastewaters. Some published studies in this particular subject include the work conducted by Pereira *et al*. (2002) in which the archaeal and bacterial clones retrieved from anaerobic mesophilic bioreactors treating oleic acid consisted of closely relatives to *Pseudomonas stutzeri*, *Desulfovibrio mexicoense*, *Syntrophomonas sapovorans*, *Erysipelothrix rhusiopathiae*, *Spirochaeta africana*, *Clostridium cellobioparum*, *Syntrophomonas* sp., *Methanobacterium formicicum*, *Methanosaeta concilli*, *Methanobacterium* sp., and also to some uncultured bacterium and archaeon.

Shigematsu *et al.* (2006) characterized an adapted mesophilic consortium that can degrade a synthetic effluent made up of oleate and palmitic acids. The acetoclastic genera *Methanosaeta* and *Methanosarcina,* the hydrogenotrophic genus *Methanospirillum,* closely related fatty acid oxidizing bacteria affiliated with the family *Syntrophomonadaceae*, and also bacteria belonging to the phyla *Bacteroidetes* and *Spirochaetes*, were detected.

Menes *et al*. (2001) characterized an anaerobic oleate-degrading consortia but in thermophilic conditions.

Sousa *et al.* (2007) have recently characterized separately the microbial communities involved in anaerobic degradation of oleic or palmitic acids. The microbial communities obtained were different from each other. The most predominant bands, obtained in the DGGE profile, from oleic acid and palmitic acid enrichment cultures, were identified. *Syntrophomonas* sp., *Chlorobium phaeobacteroids* and *Desulfovibrio aminophilus* are examples of closer related species identified in the oleate enrichment culture. On the other hand, in the palmitic enrichment culture *Syntrophomonas* sp., *Halothiobacillus* sp. and *Syntrophobacter fumaroxidans* affiliated species were detected. Additionally, Sousa *et al.* (2007d) studied the microbial communities degrading LCFA in methanogenic bioreactors and most of the bacterial clones analyzed clustered within the *Clostridiaceae*, whereas the methanogens identified were closely related to *Methanobacterium*, *Methanosaeta* and *Methanosarcina*.

Hatamoto *et al.* (2007) also studied the diversity of anaerobic microorganisms involved in mesophilic LCFA degradation in methanogenic sludges using RNA-based stable isotope probing. Two different samples were analyzed, one deriving from a lab-scale multi-staged UASB reactor and other deriving from a commercial plant. From the lab-scale reactor library, 30 clones were affiliated with *Clostridium* cluster III, 4 clones belong to candidate phylum PO11 and 4 clones clustered with phylum *Spirochaetes*. Some clones obtained from methanogenic sludges derived from the commercial plant clustered with the family *Syntrophacea* and others with members belonging to class *Deltaproteobacteria*.

In this chapter, the microbial community dynamics during the discontinuous operation of a reactor treating a synthetic dairy wastewater was assessed by denaturing gradient gel electrophoresis of PCR–amplified 16S rRNA gene fragments.

4.2 Materials and Methods

4.2.1 Biomass source

The suspended biomass used in this study was collected from a lab scale reactor operated in cycles, each one containing two phases: i) feeding, in which the reactor was continuously fed with the wastewater to be treated and ii) reaction, in which the feeding was stoped, i.e., batch conditions. Five cycles were performed during the trial period, with different combinations of organic loading rate and feeding phase time, but with a constant hydraulic retention time (HRT) of 1.6 days. In the first four cycles, 4.4 ± 0.6 $kgCOD.m^{-3}.day^{-1}$ (7.2 gCOD/l) were applied during the feeding phase. In the last cycle the organic loading rate applied during the feeding phase was increased to 8.2 $kgCOD.m^{-3}.day^{-1}$ through an increase in the concentration fed (13.1 gCOD/l). Performance data collected over time were used to define the duration of the feeding phases in each cycle. The end of the reaction phase was defined by the end of the biogas production. The reactor was fed with a synthetic dairy wastewater, composed of 50% COD-skim milk and 50% COD-sodium oleate. This substrate was supplemented with macronutrients, micronutrients and $NaHCO_3$, as described elsewhere (Zehnder *et al.*, 1980).

Table 4.1 shows the specific methanogenic activity in the presence of acetate and H_2/CO_2, determined at the beginning of reactor operation (corresponding to the inoculum methanogenic activity), in the middle of the operation (more precisely in the end of the 3rd cycle) and finally in the end of the operation (end of the 5th cycle). Note that the operating conditions applied were followed by an increase of the SMA exhibited by the sludge.

Table 4.1 Time course of SMA in the presence of acetate and H_2/CO_2 in three moments of the reactor operation.

SMA in the presence of: ($ml\ CH_{4(STP)}.gVS^{-1}.day^{-1}$)	**Beginning of the operation**	**End of the 3rd cycle**	**End of the 5th cycle**
Acetate	0	179 ± 18	246 ± 7
H_2/CO_2	56 ± 6	531 ± 10	512 ± 24

4.2.2 Nucleic acid fingerprints of microbial communities

4.2.2.1 Sample collection and DNA extraction from sludge samples

To investigate the changes in *Bacteria* and *Archaea* populations inside the reactor, five different homogenized sludge samples were taken at key moments of reactor operation, as schematized in Table 4.2.

Sludge samples were washed and centrifuged (4000 rpm, 10 min) twice in PBS (description in Appendix A). The supernatant was rejected and pellet was stored at -20ºC until further DNA extraction.

Table 4.2 Sludge sampling moments during reactor operation.

Sample	**1**	**2**	**3**		**4**						**5**
Phase and cycle	F1	R1	F2	R2	F3	R3	F4	R4	F5	R5	End of operation
Time (days)	0	17	44	62	100	118	139	167	182	204	213

F- feeding phase; R- reaction phase

Total DNA was extracted from approximately 500 µl of sample by using Fast DNA® Spin kit for soil (Q – Bio gene, Carlsbad, CA, USA), according to the manufacturer's protocol. The extracted DNA was maintained at -20ºC.

4.2.2.2 Polymerase chain reaction of 16S rRNA genes

10-fold serial dilutions from extracted DNA were made and tested in the PCR to find the best concentration of template DNA that gives a good specific product. Table 4.3 shows the volume of each reagent used in one polymerase chain reaction.

A Taq DNA Polymerase kit (Life Technologies, Gaithersburg, MD, USA) was used, which provides Taq DNA Polymerase itself in addition to PCR reaction buffer and $MgCl_2$. A specific region of the bacterial 16S rRNA gene (V6 to V8 region) was amplified using primers U968-f and L1401-r. For arqueal V2 to V3 region 16S rRNA gene amplification, primers A109(T)-f and 515-r were used.

Table 4.3 Reagents and respective volumes used in one polymerase chain reaction.

10× PCR reaction buffer (without $MgCl_2$)	5 µl
$MgCl_2$	3 µl
dNTPs (10 mM each)	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
PCR water	37.75 µl
Taq DNA polymerase (5 unities/ µl)	0.25 µl
Total volume	49 µl

A 40-nucleotide GC-rich sequence (GC-clamp) was attached at the 5'-end of the PCR primers U968-f and 515-r. Primers, CG-clamp sequences and other specifications are

indicated in Table 4.4. All the primers were synthesized commercially by MWG-Biotech, *Ebersberg, Germany*.

Table 4.4 Target sites, sequences and specificity of primers targeting small subunit ribosomal RNA used for DGGE analysis.

Primer	Target site	Sequence (5' to 3')	Specificity	Reference
A109(T)-f	109-125	ACT GCT CAG TAA CAC GT	Archaeal 16S	Grosskopf *et al*., 1998
515-r	492-515	ATC GTA TTA CCG CGG CTG CTG GCA	Universal 16S	Lane, 1991
U968-f	968-984	AAC GCG AAG AAC CTT AC	Bacterial 16S	Nübel *et al*., 1996
L1401-r	1385-1401	CGG TGT GTA CAA GAC CC	Bacterial 16S	Nübel *et al*., 1996
GC clamp		CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G		Muyzer *et al*., 1993

A volume of 1 µl of DNA template (resulted directly from the DNA extraction or from the dilutions) was added to each PCR reaction tube containing 49 µl of PCR mix (Table 4.3). The tubes were inserted into the thermocycler (Bio-Rad) and the appropriate PCR program was selected (Table 4.5).

Table 4.5 PCR-cycling conditions for the amplification of bacterial and arqueal 16S rRNA gene.

	PCR program for *Bacteria*			**PCR program for *Archaea***		
Pre-denaturation	2 min	95ºC		2 min	95ºC	
Denaturation	30 s	95ºC		30 s	95ºC	
Annealing	40 s	56ºC	35 cycles	40 s	52ºC	35 cycles
Elongation	1 min	72ºC		1.5 min	72ºC	
Post-elongation	5 min	72ºC		5 min	72ºC	
	∞	4ºC		∞	4ºC	

The size of the obtained PCR products was checked by comparison with appropriate size and mass standard (MBI Fermentas, Vilnius, Lithuania), by electrophoresis on an 1% (w/v) agarose gel. Ethidium bromide staining was applied. A negative control was also loaded in the agarose gel, which consisted of the PCR products obtained from a 49 μl PCR mix without DNA template addition. Gels ran at a constant voltage of 100 V in an agarose gel electrophoresis system (Mupid-EXU). Nucleic acids were detected in an UV transilluminator (BioRad). Reagents and solutions preparation are described in Appendix A.

4.2.2.3 Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis of PCR amplicons was performed by using the Dcode system (BioRad, Hercules, CA, USA).

Assembling the DGGE plate cassette

The large and the small glass plates and the spacers were first cleaned with soap and after with 96% ethanol. The gelbond (Cambrex Bio Science, USA) was fixed, with some drops of water, to the largest glass plate. The spacers were placed on the gelbond and then the small glass plate was placed over the spacers. Clamps were added to the sides of the plate cassette and placed in the cassette-holder for glass plates, spacers and clamps adjustment.

Casting the denaturing gradient gels

The gel solutions (HIGH, LOW, plug and stacking gel) were prepared in 50 ml tubes, on ice, as described on Table 4.6. For archaeal amplicons analysis, a 30-50 % gradient was used whereas bacterial amplicons were analyzed in a 30-60 % denaturing gradient.

Table 4.6 Denaturing gradient gel electrophoresis mixing table.

Solution	Gradient %	0% solution ml	100% solution ml	TEMED µl	10% APS µl
Stacking gel	0	9	-	13	50
LOW	30	9.1	3.9	13	50
HIGH	50	6.5	6.5	13	50
HIGH	60	5.2	7.8	13	50
Plug gel	-	1.5	-	4.5	15

A volume of 1 ml of plug gel solution (Table 4.6) was poured at the bottom of the DGGE plate cassette (between glass plates) prior to make the main resolving gradient gels, preventing leakage from the bottom of the plates.

For casting gradient gels, a gradient former, a magnetic stirrer and a peristaltic pump (BioRad) were used. The tube exiting from the peristaltic pump was attached between the glass plates. The HIGH solution was placed into the outflow chamber of the gradient former and the LOW solution into the other chamber. The solutions were kept under continuous agitation. The valve of the gradient former was opened and the peristaltic pump turned on. The gel was formed in the plate cassette at a flow rate of 4 ml/min. The stacking gel was poured in the same way and the comb was further applied between the glass plates.

Running the gel

The buffer tank was filled with 0.5x TAE buffer and the temperature control was set at 60ºC. After polymerization of the gel, the comb was removed and the slots were rinsed with buffer. The DGGE plate cassette was placed into the buffer tank. PCR amplicons were mixed with loading buffer (4:1) and loaded onto the gel slots. The power supply was first switched at 200 V for 10 min. Electrophoresis was performed at a constant voltage of 85 V for 16 hours.

Silver Staining of DGGE gels

The silver staining of DGGE gels was performed as previously described by Sanguinetty *et al.* (1994) with minor modifications.

The gel was removed from the electrophoresis apparatus and placed in a stainless steel box.

The following steps were applied to the gel, under continuous agitation:

- Incubation in 200 ml of 1x Cairn's fixation solution, 3 min;
- Incubation in 200 ml of silver staining solution, 10 min;
- Immersion in dH_2O for 2 min;
- Immersion in developer solution until the gel has developed;
- Incubation in 1x Cairn's fixation solution, 5 min;
- Incubation in Cairn's preservation solution, 7 min;
- A pre-wet cellophane foil was placed on the gel;
- The gel was dried overnight at 60ºC.

Reagents and solutions preparation are described in Appendix A.

Analysis and comparison of DGGE community profiles

DGGE gels were scanned at 400 dpi and DGGE profiles were compared using the GelCompar II software package, which is an example of computer-assisted guideline for the analysis of fingerprinting profiles.

Similarities between banding patterns, taken in pairs, were expressed as a percentage value of Dice similarity coefficient. Cluster analysis was performed by the unweighted pair group method with arithmetic averages (UPGMA) with the aim of identifying the samples which generate similar patterns.

4.3 Results and Discussion

In order to assess the microbial communities of anaerobic treatment processes, classical parameters such as VS, microscopic observation and culture based counts are normally used, being informative but not sufficient. An important parameter, to assess the efficiency of anaerobic treatment systems is the methanogenic activity, which can be determined by measuring the SMA. However, none of these parameters can explain the failure of biological treatment systems which often remain unexplained, partially due to lack of information about the constituent microorganisms (Akarsubasi *et al.*, 2005).

In this work, the genetic diversity of bacterial and archaeal communities, developed during the operation of an anaerobic reactor treating a synthetic dairy wastewater, was studied in specific moments of the reactor operation (Table 4.2), by means of DGGE, after amplification of the archaeal and bacterial 16S rDNA genes (Figure 4.1). This anaerobic consortium exhibited an increasing SMA in the presence of acetate and H_2/CO_2 along time (Table 4.1) and the overall performance of the treatment process was improved (Cavaleiro et al., 2007) probably due to the development of an important consortium, acclimated to LCFA.

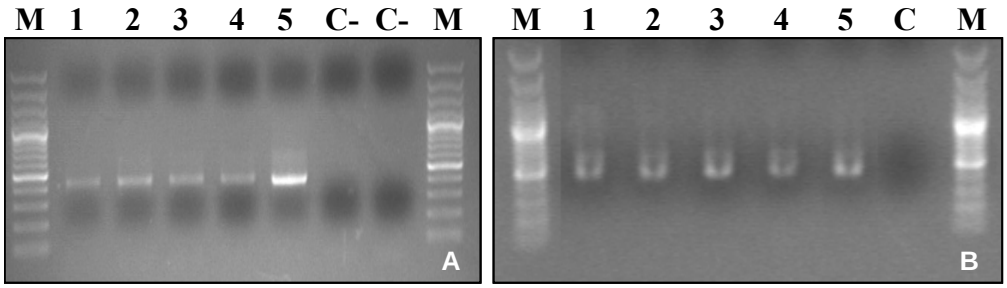


Figure 4.1 A, PCR amplification of the archaeal 16S rRNA gene (V2 to V3 region) using primers A109(T)-f and 515-r. **B**, PCR amplification of the bacterial 16S rRNA gene (V6 to V8 region) using primers U968-f and L1401-r. Marker reference bands (1000 and 500 bp) are the more intense.

According to the DGGE profiles (Figure 4.2), the number and position of bands exhibit some changes, suggesting temporal modifications of the structure of archaeal and bacterial communities. Cluster analysis of the band-patterns was performed and the resulting dendrograms and distance matrixes are shown in Figure 4.3 and 4.4, respectively.

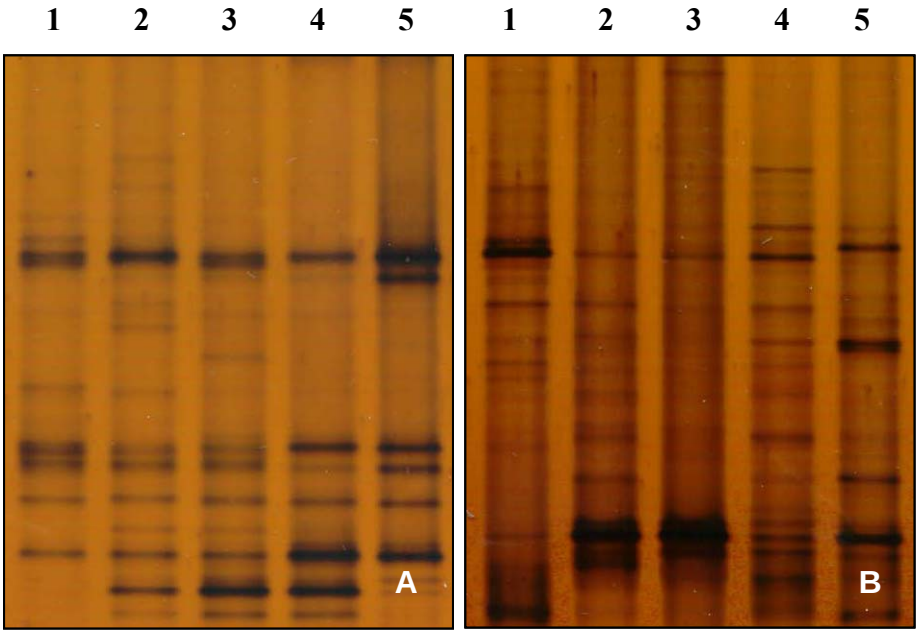


Figure 4.2 Silver-stained PCR-based DGGE fingerprints of *Archaea* (A) and *Bacteria* (B) based on extracted DNA from samples collected from the reactor in different sampling moments. Lane 1: inoculum; Lane 2: end of the feeding phase of the 1st cycle; Lane 3: end of reaction phase of the 1st cycle; Lane 4: end of the 2nd cycle; Lane 5: end of operation.

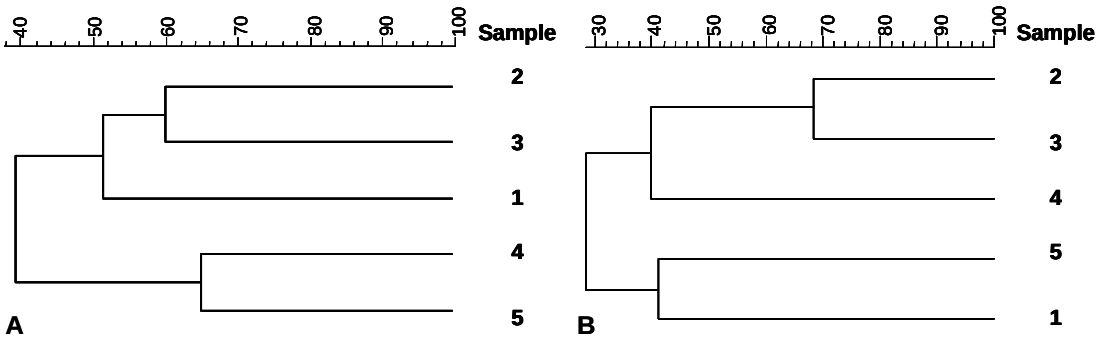


Figure 4.3 Cluster analysis by the unweighted pairwise grouping method with mathematical averages (UPGMA, Dice coefficient of similarity) of DGGE profiles of archaeal (A) and bacterial (B) amplicons.

Bacteria						**Archaea**					
Sample						**Sample**					
2	100					**2**	100				
3	68.4	100				**3**	59.5	100			
4	44.4	35.3	100			**1**	51.3	50.0	100		
5	35.0	26.3	27.8	100		**4**	30.3	40.0	37.5	100	
1	25.6	27.0	28.6	41.0	100	**5**	32.4	51.4	37.8	64.5	100

Figure 4.4 Distance matrix; Pairwise similarities (%) between the profiles obtained for each sample.

The cluster analysis of the banding patterns of *Archaea* (Figure 4.2 A) shows that samples are divided in two main clusters, namely the samples taken at the beginning of the operation (inoculum and first cycle, after a feeding and reaction phase), and those obtained in the middle and at the end of the operation (end of the 2nd cycle and end of the 5th cycle). Consecutive sampling points clustered together (e.g. 2 and 3, 4 and 5). Furthermore, the patterns of sample 1 (inoculum) and samples 2 and 3 (corresponding to the middle and the end of the 1st cycle) exhibited similarity values higher than 50%. These results point out that, during the time course of the reactor operation, a specialized consortium was gradually developed and the major shift in the archaeal community was verified during the 2nd cycle, since the similarity index among the DGGE band-patterns of sample 4 and 5 is relatively high (64.5%) when comparing the same samples with samples 1 to 3 collected in the first cycle. As indicated by the obtained similarity indexes, all the band-patterns seem to be considerably different from each other (note that the highest similarity index is 64.5), suggesting that the archaeal community was changing continuously along the trial period (Figure 4.4).

These facts agree well with the performance data of the reactor. In the first two cycles, 70% of the methane-COD was produced in the reaction batch phase, whereas from the third to the fifth cycles biogas production in the reaction phase was less than 3% of total production. LCFA accumulated onto the sludge in the first two cycles while in the subsequent cycles no LCFA were detected (Cavaleiro *et al.*, 2007). As stated before (Table

4.1), the specific methanogenic activity in the presence of acetate and H_2/CO_2 increased significantly along the operation, particularly between time zero and the end of the third cycle.

Some bands in the DGGE patterns were maintained until the end of the operation and seem to correspond to the dominant species, because they show a higher intensity when compared with the remaining bands. Changes in the intensity of the different bands suggest variations in the relative abundance of the different 16S rDNA defined populations (Lebaron *et al.*, 1999). However there is always some bias occurring in the whole extraction-amplification procedure of the genomes that have to be considered. In order to minimize the errors on doing this assumption, the DGGE analysis should be restricted to samples treated using the same methods, in which DNA extraction and amplification biases are supposed to occur homogeneously (Fromin *et al.*, 2002). All samples analyzed in this study were treated in the same conditions. Additionally, after the amplification of the same DNA template in different PCR reactions, the DGGE fingerprint seems to be reproducible (data not shown).

In the archaeal DGGE fingerprints (Figure 4.2 A), the intensity of some bands increased with time, suggesting that some species were not dominant at the beginning of the operation, but had some characteristics that allowed the persistence in the system when the operation conditions changed. Species which were always present from the beginning until the end of the reactor operation, may be the ones that are better adapted to the conditions applied and are probably more resistant to high concentrations of LCFA than the others. It was also found that some bands disappeared and others have suddenly appeared and continued present until the last sampling time (Figure 4.2). The remaining may represent some organisms that were not present in the inoculum, or could be present but were not detected, and were then able to grow probably due to the changes of reactor conditions and/or to the disappearance of other organisms which could be competing for the same substrate. The bands that disappeared probably correspond to species that were not able to survive/grow in this kind of environment.

According to the fingerprint results, the change of the feeding regime during the reactor operation (the increase of the organic loading rate from 4.4 to 8.2 kg $COD.m^{-3}.day^{-}$

[1]) seems to be an important factor influencing the genetic diversity of the archaeal community.

The cluster analysis of the DGGE band-patterns of *Bacteria* (Figure 4.3 B) resulted, also, in two main clusters. However, the similarity values among samples are quite low. Sample 1, i.e. inoculum, and sample 5, i.e. collected at the end of operation, clustered together, but the similarity value between the two samples is of only 41.0 % (Figure 4.4). The same happened with the remaining three samples (2, 3 and 4) that clustered together, but exhibit a similarity index of approximately 40 % (Figure 4.3 B). The highest genetic proximity was verified between sample 2 and 3, collected in the first cycle after a feeding and reaction phase, respectively, which exhibited a similarity of 68.4%.

The number of apparent bands, in the bacterial DGGE fingerprints, were constant among the samples collected (20 to 21 bands), but considerable differences were observed in the positions and intensity of the bands (Figure 4.2 B).

The obtained results suggest that the bacterial community was more affected by the conditions imposed within the reactor than the archaeal one. It also seems that the shifts in bacterial community resulted in an adapted consortium able to efficiently treat this kind of wastewater, since overall COD removal efficiency increased steadily along time (from a minimum value of 71 % to a maximum value around 98 % in the last two cycles) and the specific methanogenic activity in the presence of acetate and H_2/CO_2 increased significantly (Table 4.1) during reactor operation (Cavaleiro *et al.*, 2007).

At the end of the operation (sample 5), more intense bands are seen, suggesting that the existent species may also be present in a high amount and probably play an important role in the degradation of LCFA.

In order to support the suggestions stated above, further identification and investigation of the species should be conducted. The presence in these samples of the recently identified and isolated *Syntrophomonas zehnderi*, that degrades LCFA up to 18 carbon atoms, was investigated. However, there was no evidence of its presence in the samples analyzed and probably other organisms capable of degrading the same substrates were present. Further work should be done in order to get more insights about the diversity of anaerobic LCFA degrading consortia.

In a previous study, Pereira *et al.* (2002) monitored the microbial diversity of granular and suspended sludge treating oleic acid and reported that the bacterial community was also more affected by the increase of the organic loading rate than the archaeal community. An organic loading rate of 6 kg $COD.m^{-3}.day^{-1}$with oleate as the sole carbon source was suggested by these authors as the maximum value that could be applied to the system. As reported by Cavaleiro *et al*. (2007), an organic loading rate of 8.2 kg $COD.m^{-3}.day^{-1}$ (oleate/ skim milk 50:50) was applied and efficiently converted to methane, with no SMA reduction, by the microbial consortium studied in the present work.

5 General Conclusions and Perspectives for Further Research

Oleic acid degradation was studied in batch assays and the dynamics of the microbial population developed in a lab scale reactor, treating an oleic acid based effluent, was also investigated by analysis of the 16S rRNA gene using the polymerase chain reaction (PCR) based denaturing gradient gel electrophoresis (DGGE) method.

The following conclusions can be made based on the obtained results:

1. A biomass highly loaded with LCFA, exhibiting low methanogenic activity was severely inhibited by the addition of oleate concentrations in the range of 500 to 1500 mg/l. However, after degrading the adsorbed substrate, the biomass performance improved considerably since the methanogenic activity increased 2 and 2.6 times in H_2/CO_2 and acetate as individual substrates, respectively. Also, the ability to degrade oleate, in the same concentration range, was enhanced, since lag phases preceding the initial production of methane decreased considerably and methane recovery increased from 50 to 80%.

2. The recovery of the methanogenic activity after the degradation of the accumulated substrate (LCFA) was more evident when higher amounts of biomass associated substrate (3000 to 6000 mg COD/g VS) were present.

3. The intermediates of oleic acid anaerobic degradation were identified, in batch assays performed with two different biomasses, one previously acclimated to LCFA and other non-acclimated. The accumulation of high amounts of LCFA intermediates was not verified and only a transient acetate accumulation was observed. Within the first 50 minutes almost all oleate was adsorbed onto the biomass and after a typical lag phase, about 830 mg/l of oleate were converted to methane by both sludges. The acclimated biomass was able to degrade the added oleate in 5 days whereas the unacclimated one took around 12 days.

4. During the anaerobic treatment of a synthetic dairy wastewater the shifts on the composition of bacterial and archaeal communities, revealed by DGGE analysis of

PCR amplified 16S rRNA fragments, were monitored. Bacterial community was more affected by the conditions imposed within the reactor than the archaeal one.

This work contributes to a better understanding of the most suitable conditions for an efficient LCFA mineralization, under anaerobic conditions. However, further research is needed concerning this particular subject, in order to improve the knowledge of the microbiological and biochemical mechanisms of LCFA degradation. It would be interesting to follow the intermediates accumulated in the solid phase, as described in Chapter 3, but during the mineralization of biomass associated LCFA by a heavily loaded sludge, similar to the one studied in Chapter 2. Since microorganisms are responsible for the degradation of LCFA and only few species are already isolated and characterized, it is important to continue the study of the communities developed in such environment, especially the consortia implicated in efficient treatment processes like the one studied in Chapter 4.

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Appendix A

Solutions and Reagents

LCFA analysis

HCl (25%): 1-propanol solution:

Add 25 ml of HCl (37%) to 75 ml of 1-propanol.

Internal standard solution:

Dissolve 0.1 g of pentadecanoic acid (C15) complete to 100 ml with dichloromethane.

Calibration curve:

The calibration curves were prepared with the following acids: lauric (C12), myristic (C14), palmitic (C16), palmitoleic (C16:1), stearic (C18), oleic (C18:1) and linoleic (C18:2).

A quantity of 0.1 g of each LCFA was added and the volume was adjusted with dichloromethane to 100 ml. Successive dilutions were made with DCM to obtain solutions with the following concentrations: 100, 250, 500 and 750 mg/l. In glass tubes, 2 ml of each solution, 1.5 ml of internal standard solution, 1.5 ml of HCl:1-propanol solution and 2 ml of Milli-Q water were added. LCFA extraction and analysis was performed as described in section 2.2.2.2.

Molecular Biology analysis - DNA extraction, PCR and DGGE

Phosphate buffered saline (PBS)

- 0.8 g NaCl
- 0.02 g KCl
- 0.144 g $Na_2HPO_4.2H_2O$
- 0.024 g KH_2PO_4

Complete to 100 ml with Milli-Q water and adjust to pH 7.4. Filter with a sterile filter (0.2 μm). Autoclave 15 min at 121ºC.

PCR Water

Filter Milli-Q water with sterile filters (0.2 μm) and autoclave (121ºC, 15 min). Distribute aliquots of 1 ml into sterile eppendorfs in a PCR/UV work station. Store at -20ºC.

1% (w/v) agarose gel

- 75 ml TAE 0.5x
- 0.75 g agarose
- 3 μl ethidium bromide

Dissolve agarose in microwave oven. Cool the agarose to 55-65 ºC and add ethidium bromide.

50x TAE buffer (pH 8.3)

- 242 g Tris base
- 57.1 ml acetic acid, glacial
- 100 ml EDTA 0.5 M (pH 8.0)

Adjust to 1 liter with dH_2O. Autoclave for 20 minutes. Store at room temperature.

DNA loading buffer

- 46.8 ml glycerol 80%
- 76.7 ml dH_2O
- 1.5 ml EDTA 0.5 M
- 0.15 g bromophenol blue
- 0.15 g xylene cyanol FF

dNTP solution

- 100 µl each dNTP (from 100 mM dNTP set)
- 600 µl PCR water

Distribute 100 µl to 10 sterile eppendorfs.

Table A. 1 Denaturing solutions preparation.

Reagents	**0% Denaturing solution**	**100% Denaturing solution**
40% acryl/bisacrylamide 37.5:1 (BioRad)	200 ml	200 ml
50x TAE buffer	10 ml	10 ml
glycerol	20 ml	20 ml
formamide	-	400 ml
urea	-	421.6 g
dH_2O	to 1000 ml	to 1000 ml
Store in the dark at room temperature.		

10% Ammonium persulfate (APS)

- 1g ammonium persulfate
- 10 ml dH_2O

Store at -20 ºC in 500 µl aliquots.

TEMED

- N, N, N', N'- Tetramethylethylenediamine ready-to-use solution.

Cairn's 8x fixation solution

- 200 ml 96% ethanol
- 10 ml acetic acid
- 40 ml dH_2O

Silver staining solution

- 0.4 g $AgNO_3$
- 200 ml of 1x Cairn's fixing solution

Developer solution

- a spatula tip of $NaBH_4$ (approx. 10 mg)
- 250 ml 1.5% NaOH solution
- 750 µl formaldehyde

Cairn's preservation solution

- 250 ml 96% ethanol
- 100 ml glycerol
- 650 ml dH_2O